ANNUAL REPORT OF PROGRAM ACTIVITIES NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES FISCAL YEAR 1974

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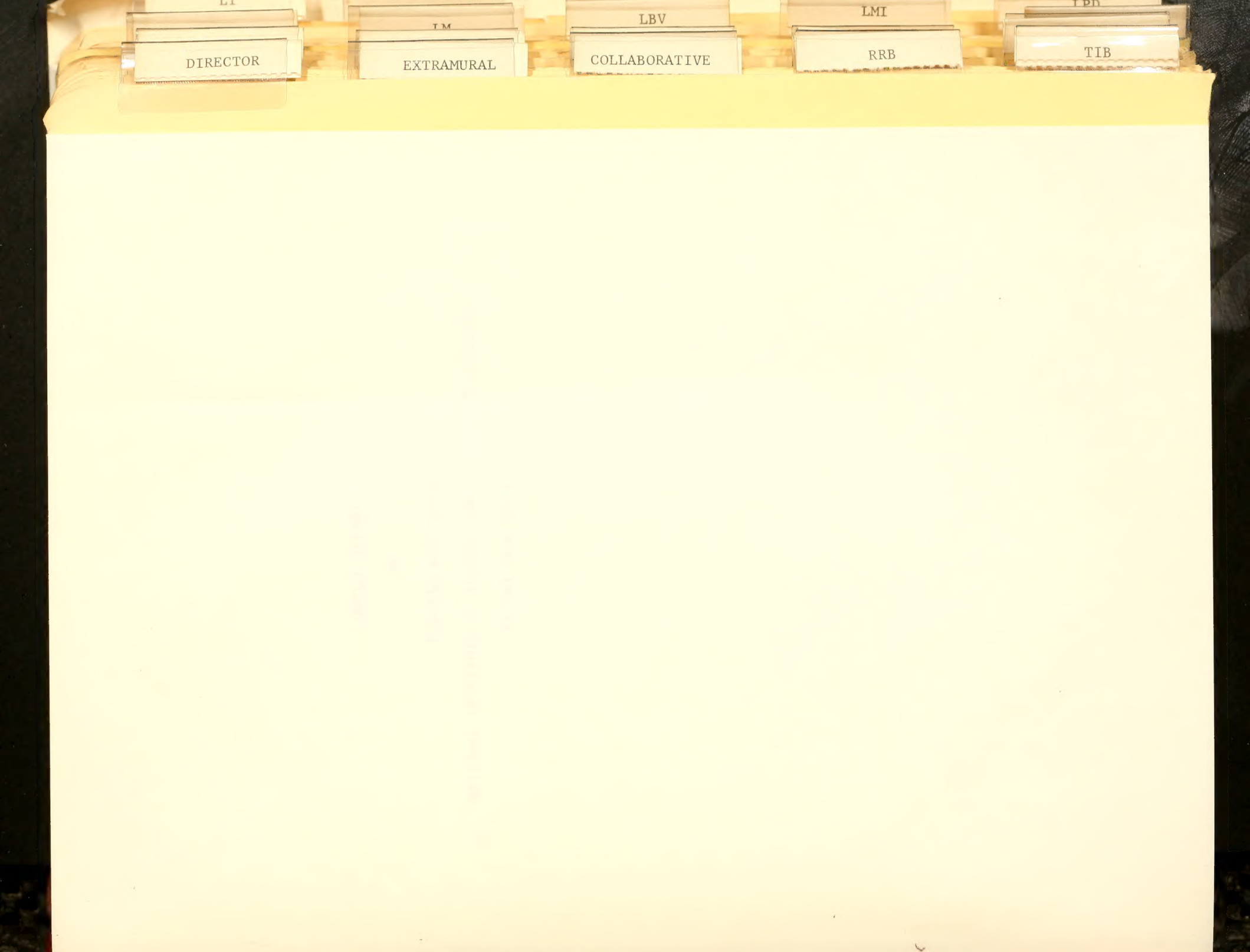
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ANNUAL REPORT

OF

PROGRAM ACTIVITIES

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

Fiscal Year 1974

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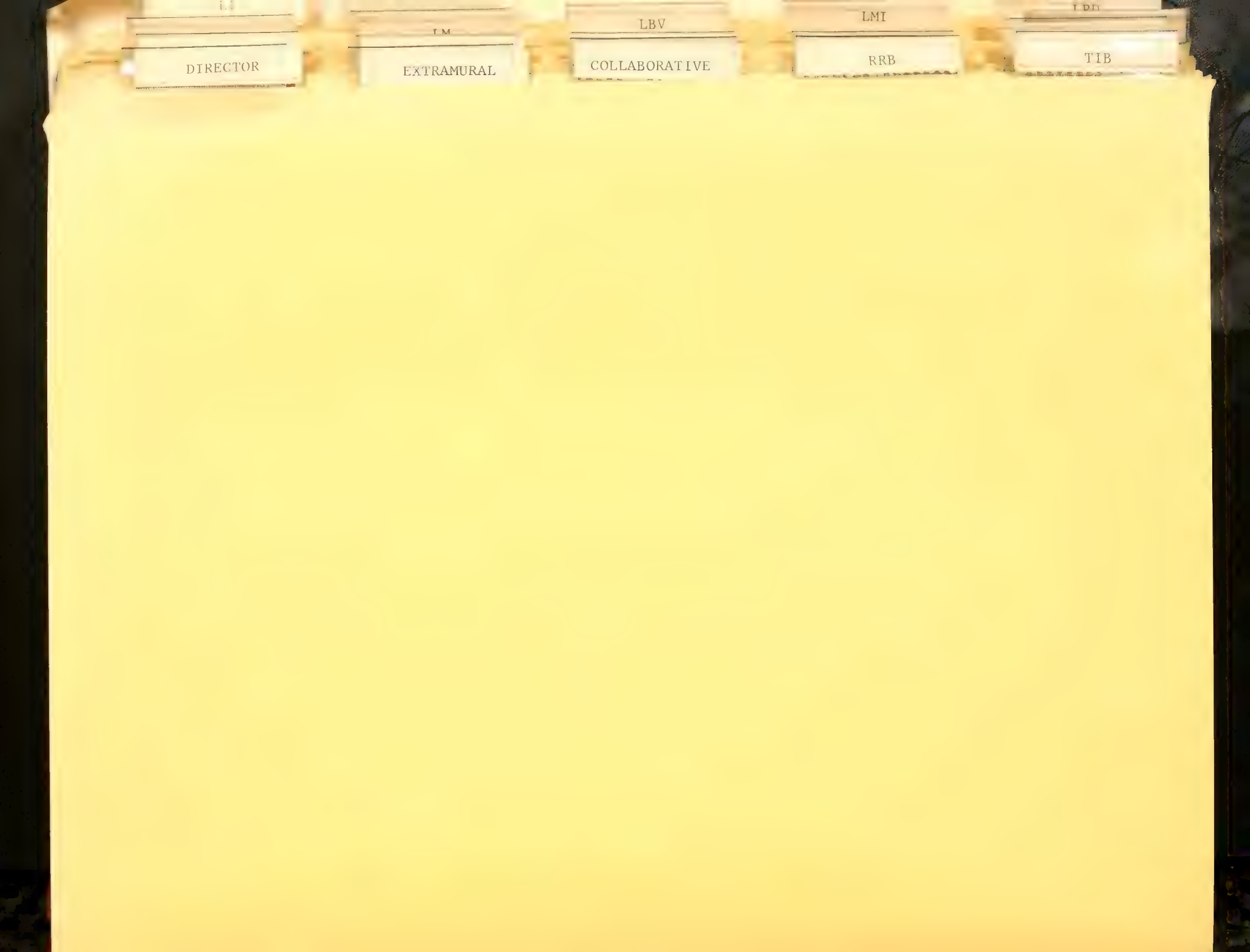
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ANNUAL REPORT OF PROGRAM ACTIVITIES
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
JULY 1, 1973 THROUGH JUNE 30, 1974

The National Institute of Allergy and Infectious Diseases conducts and supports a broad program of research directed at improved ways of diagnosing, treating, and preventing allergic conditions and infectious diseases.

A feature of the extramural grants activities was the uncertainty of the form of research training programs. The adjustment to the new method of support required a continual vigilance to respond to applicants and grantees of research training grants within the framework of new plans.

The requirement to reduce the staff during this year presented major management problems for the intramural programs. The Institute plan to meet this requirement was by phasing out some laboratory projects and reassignment of both professional and supporting personnel. Difficulties were encountered in making appropriate reassignment, and the attempt to maintain desired capability without attrition was not fully accomplished.

Problems of assurance of protection of human subjects has appeared in all the areas of the Institute's research programs. The NIH procedures and guidelines now being developed are expected to provide more uniformity in acceptance by concerned groups and in this sense permit better planning for the pursuit of research objectives in the future.

The Institute has been authorized to enlarge the National Advisory Allergy and Infectious Diseases Council membership by three for a total of 15 members. This action was requested in order to broaden the scientific competence of the Council and particularly in the areas of allergy and immunological diseases. The programs and subject matter of applications have become more complex and varied in the 19 years since the Council was established.

New knowledge is of limited value unless it can be communicated promptly and effectively to those who need or can use it. Therefore, the Institute program for support of scientific communications, and development of improved methods, has kept apace with support of research programs.

The largest portion of communications funding related to the publication of formal research manuscripts; their preparation, page costs for printing, and a new item of cost initiated by one of the journals -- manuscript review costs. Manuscripts and research notes related to NIAID programs and submitted for publication in FY74 were approximately as follows: 3,000 from grantees, 200 from contractors, and 425 from the Institute staff. In addition, a number of research reports, monographs and scientific meeting

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reports were printed with total or partial funding from the Institute. Copies of the technical reports were sent to the Department of Commerce "National Technical Information Service" for availability to the scientific community and the public.

Second in size of funding is support of scientific meetings; either in total or in part. Support was provided in FY74, in some instances as co-sponsorship with other NIH Institutes, for six major meetings and one workshop. These represented about 50% of the applications. In addition, there were 17 workshops or small conferences conducted as part of individual program activity, and 54 seminars were held at NIAID during the year. The trend continued in an increasing number of workshops as compared to large scientific meetings. The concentrated discussions on a single subject or a narrow area of research afford indepth communications of great value to research and program progress.

The program activity devoted to development of new and improved methods of scientific communications progress satisfactorily. The mechanism for informal communications of pre-published information on current research investigations is known as the "Scientific Memoranda." Projects continued for investigators involved in research on interferon, hepatitis, leprosy, hypersensitivity disease, neisseria-treponema, and transfer factor, with distribution of communications either monthly or quarterly. Participants in these projects number almost 1,000, located in 53 countries, including for the first time this year the Peoples Republic of China.

Feasibility studies continued on the use of satellites for scientific communications with research investigators at isolated sites or while on field studies. Using the NASA ATS-1 satellite over the Pacific Ocean and a transmission period of half to one hour per day, an increasing number of investigators were able to discuss research protocols, experimental results, and field observations with colleagues and consultants in other parts of the Pacific Ring and in various areas of the United States. The satellite permits direct and frequent scientific discussions between investigators in situations where other means of communications are impractical.

In line with a new policy of the DHEW Office of Public Affairs, responsibilities for "public affairs" were transferred from NIH's Bureaus/Institutes/Divisions to its central Office of Public Affairs and the number of personnel in public affairs and publications dollars were reduced. As a result of this reorganization at NIH, the NIAID Research Information Section was re-designated as the Office of Research Reporting and Public Response (ORRPR), and the Institute lost one professional position and $22,000 in publications costs. The ORRPR continues to respond to information requests from the general and specialized publics and to produce research reports and publications which help carry out this function.

A series of publications on allergies was initiated this year, and single copies of the first three pamphlets -- "Food Allergy," "Dust Allergy," and "Pollen Allergy" -- were distributed to the Nation's allergists as suggested aids for communicating with their patients.

"Immunology Research, An Introduction" was distributed to allergists and immunologists. This booklet received numerous favorable comments, particularly from immunologists, who plan to use it in educating medical students and technicians.

Two publications -- "Bacteria: The Littlest Cells" and "Viruses: On the Border of Life" --were translated into Spanish for distribution to Spanish-reading Americans. "Sinusitis" and "Flu" were published in the series on infectious diseases established last year with the production of a pamphlet on "Hepatitis". Two new fact sheets --"Tuberculosis" and "Tetanus" -- were added to the Office's series of fact sheets on infectious disease research; three others -- "Fungal Infections," "Mycoplasma," and "Interferon" --were updated. The Office now distributes 21 different fact sheets.

The Institute co-sponsored a one and one-half hour long-distance telephone briefing between allergists at the annual meeting of the American Academy of Allergists in Bal Harbour, Florida, and science writers gathered in the NIAID conference room at Bethesda. Ten scientists discussed original papers, and manuscripts were available at Bethesda for the briefing. This successful briefing demonstrated the value of using long-distance conference calls to save costs and time, particularly in light of the energy crisis. A small exhibit at the Academy meeting displayed Institute publications on immunology and allergic diseases.

Statistics were developed on morbidity and mortality caused by infectious and allergic diseases, and on their economic impact in terms of direct and indirect costs. A paper was prepared on "Measurement of the Prevalence of Respiratory Allergies by Interview Questionnaire," and submitted for publication. Progress was made toward completion of a second paper on "Age at Onset of Asthma and Allergic Rhinitis in the Tecumseh, Michigan Population." Consultation on a variety of statistical matters was provided to the Institute Director, the Office of Research Reporting and Public Response and other NIAID staff members. Project Officer functions were carried out in connection with two contracts: one with the University of Florida,College of Medicine, and the other with the Kaiser Health Plan in Portland, Oregon.

During the past year there were some significant developments in the framework used by Institute staff to conceptualize Institute programs and activities. The framework used for formal presentation and justification of the annual budget request underwent a radical revision with the mandated move to a Program Budget structure. This is in turn crystallized a program structure that had been under discussion and development by staff for several years. The current program structure is presented in Table 1.

Items 1.0 through 6.0 are Institute Programs (IN CAPITALS) including research and training projects. Collectively they account for 100% of the Institute budget. These are supported by a mix of 3 mechanisms (Extramural Grants and Awards, Collaborative Contracts and L&CR Projects).

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For purposes of program definition; presentation of accomplishments to public, legislative, or management audiences; and budget explanation and presentation the Institute Staff has agreed upon a set of Institute Subprograms.

A Subprogram by definition is a portfolio of research projects that may be supported by a mix of the 3 support mechanisms and has either M1, M2 or M3 degree of management. The highest degree of management (M) or Federal intervention is indicated by 3.

The purposes of an M3 portfolio are relatively tightly defined and identifiable. The portfolio is characterized by most of the following criteria:

has one or more program officers

includes a contracts component

seeks research solutions to a major national public health problem

is the subject of special legislative interest

includes a structured communications activity

includes an Extramural Special Emphasis Program

Embedded in an Institute Subprogram may be special programs peculiar to one mechanism of support, such as the Extramural Special Emphasis Programs (SEP).

The M2 portfolios at present are exclusively Extramural and specifically include the relevant SEP portfolios; although there is no scientific reason why relevant Intramural projects could not be included in an SEP. These M2 SEP exemplify a lesser degree of management. They are characterized by having a part-time program officer, and a formal, brief program statement which includes goals and content. The statements are regularly reviewed by the National Advisory Council and the program content, size and balance considered when Council recommends (at least annually) a dollar allocation to the program area (DAPA). The latter device permits a somewhat increased and more timely level of effort by making awards for applications slightly beyond the priority score level at which grants are regularly being paid.

The SEP concept then serves as a focus:

a) for presentation to the legislative public

b) for staff programming for increased effort

c) for directing Council's attention to broad program concerns rather than individual projects

d) for providing a mechanism for early preferential funding.

Additionally, it serves as a focus for program analysis and evaluation activities. The Institute has started a cycle of SEP evaluation by ad hoc expert groups. Three SEP have been evaluated sofar and a fourth is scheduled. The technique for such evaluations is being refined in the course of this cycle.

The evaluation activity and the evaluation group report serve as an invaluable communications mechanism (complete with feedback loop) with the scientific public.

A number of criteria may enter into the selection of a research area for special emphasis. These selection criteria include:

1. Public health importance of the Disease/Disease Problem (D/DP)

    a) Demonstrable (recognized or established) by morbidity - mortality statistics or socioeconomic impact

    b) Conjectural - expert opinion regarding generally unappreciated or unrecognized major problem

2. State of the Art

    a) Possibility of rapid progress, including exploitation of breakthrough

    b) Overlooked area because of interdisciplinary communication gaps, disciplinary vogues

    c) Broad implications for biomedical research

    d) Specific knowledge deficiencies

3. Inadequate resources causing neglect of area

    a) Techniques and methods

    b) Facilities

    c) Unusual expenses

    d) Unusual requirement for cooperation

4. Ecology of Science considerations

    a) National requirement for maintaining a cadre of competence

    b) Type or degree of national support (e.g, amount or balance)

    c) Institutional or geographic considerations

    d) Availability of manpower

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Special Emphasis Programs are established only after an extensive dialogue between staff, Council and other advisors culminating in an Institute decision and acceptance of a program statement.

Although an SEP that is included in an M3 portfolio has essentially the same degree of management as one in an M2 portfolio, it should not be concluded that categorization in an M3 portfolio is for trivial reasons. An SEP so included is, of course, immediately relevant to the scientific concerns of the Subprogram; but more than that, to the population of scientists who comprise the SEP principal investigators and their colleagues. This population is a valuable program resource. It probably: a) provided the knowledge base on which the Subprogram was founded, b) is a source of experts needed to review contract proposals, c) serves as a source of new contractors for accomplishment of particular program goals and d) acts as a communications network for dissemination of information concerning program goals, accomplishments and problems.

The majority of the Institute supported activities are included under the M1 portfolios of the five Scientific Programs (Table I). These portfolios include research and training awards and projects concerned with numerous diseases and disease related problems relevant to the Institute's mission. The projects represent the best judgments of over 4000 scientists as to what the most significant problems are and the techniques and approaches most apt to yield new and important knowledge or tangible results. In the aggregate they cover an astounding array of diseases, organisms, problems and expertise. In their diversity and high quality lies their short- and long-range value. Quite properly this creative activity remains unmanaged as to science and therefore untargeted and unfettered except in the most general sense of relevance to the Institute mission.

Both the program and the constituent projects are subject to continuing activities dealing with statements of program content or description, review for relevance, and merit; while the program is reviewed for content, goals, objectives, and feasibility. Table II uses one Program--Viral Diseases --as an example of who performs these activities.

TABLE 1

NIAID PROGRAMS AND SUBPROGRAMS

Program	M1 Portfolio	M2 Portfolio	M3 Portfolio
1.0	ALLERGIC & IMMUNOLOGIC DISEASES	Selective Activation of Cell Mediated vs. Humoral Antibody Responses	Asthma and Allergic Disease Centers
		Lymphocyte Biology	Clinical Immunology
2.0	BACTERIAL & FUNGAL DISEASES	Mechanisms of Resistance to Antimicrobial Agents	Sexually Transmitted Diseases
			Diseases Preventable by Vaccines
			Hospital Associated Infections
3.0	VIRAL DISEASES	Clinical Virology	Influenza and Other Respiratory Infections
		Chronic and Degenerative Diseases of Man	Hepatitis
			Antiviral Substances
4.0	PARASITIC DISEASES	Immunity to Parasites	
		Biological Regulation of Vectors	
5.0	INTRAMURAL LABORATORY & CLINICAL RESEARCH (includes relevant Mgmt. Fund)		
6.0	RESEARCH MANAGEMENT AND PROGRAM SERVICES (includes relevant Mgmt. Fund; Review and Approval; Program Direction; and Collaborative Support)		

TABLE II

VIRAL DISEASES--AN NIAID PROGRAM

	M1 Portfolio	M2 Portfolio	M3 Portfolio
PROGRAMS/ SUBPROGRAMS	Research approaches to specific Diseases and Disease Related Problems as perceived by individual investigators	Clinical Virology Chronic & Degenerative Diseases of Man	Influenza and Other Respiratory Infections Hepatitis Antiviral Substances
PROGRAM CONTENT OR DESCRIPTION	See Awards Book. Out of 122 terms used to categorize the Institute's programs some 22 deal with virus diseases and related problems	See Special Emphasis Program statements and periodic evaluation reports	See numerous periodic and ad hoc documents
REVIEW FOR RELEVANCE	Institute Grant Referral Guidelines DRG (Council)	Council Subcommittees	RCAG Council Subcommittees
REVIEW FOR MERIT	Study Sections (Council) Lab Chiefs (IM)	Study Sections (Council)	Subcommittees Ad hoc groups including IM/NIH
REVIEW OF PROGRAM CONTENT, GOALS, OBJECTIVES, FEASIBILITY	Board of Scientific Counselors (IM)	Council Subcommittees Staff Ad hoc evaluation groups Scientific Director & Board	Senior Staff Advisory Committees Interagency Liaison
FOCUS OF MANAGEMENT AND INTERVENTION	Lab Chiefs (IM) Scientific Director (IM)	Program Officers (EP) Scientific Director (IM)	Program and Project Officers Senior Staff

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EXTRAMURAL PROGRAMS - NIAID

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ANNUAL REPORT OF PROGRAM ACTIVITIES

National Institute of Allergy and Infectious Diseases
July 1973 - June 1974

I. REPORT OF THE ASSOCIATE DIRECTOR

Another year with unusual fiscal experiences has just passed. The most unusual feature of Fiscal Year 1974 was the result of Court Order 1830-73 from the U.S. District Court which released "impounded" funds for Fiscal Year 1973. This resulted, as shown in Table I, in the funding of approximately 50 new and renewal research awards and restoration of funds to about 80 research awards.

The result of this Court Order was also a continuation of the training grant and fellowship program for which the funding of competing awards had terminated in mid-Fiscal Year 1973. Approximately 16 new and renewal training grant awards and 14 fellowship awards were made as a result of this release of impounded funds. At the same time, staff was responsible for reviewing about 345 individual and 42 institutional research fellowships and supporting slightly over 100 fellows included in 5 institutional and 87 individual fellowships.

It is especially noteworthy that a vigorous and devoted staff accomplished all of these actions with a smaller work force than was available for the latter half of Fiscal Year 1973.

The Institute's Extramural Program continued to expand its efforts in the Asthma and Allergic Disease Centers, Venereal Disease, and Hospital-Associated Infections. The Hospital-Associated Infections Program, which was started in late FY 1973 with a small nucleus of ongoing research grants, has accomplished considerable expansion through new research projects programmed by EP staff. Influenza and Clinical Virology were also designated as Special Emphasis Programs within the past year. However, since they were designated late in the year trends of these programs will not be possible to predict until next year. Our investment extramurally in infectious hepatitis was slightly reduced in FY 1973 and "Mechanisms of Immunity to Infectious Agents" was discontinued as a Special Emphasis Program. Over all, there have been some increases in the support of research on allergic and immunologic diseases, and smaller increases for viral diseases, with the research in the bacterial and fungal disease areas and the parasitic and arthropod-borne disease areas holding at a rather stable level.

Table I describes overall data relevant to FY 1973 and FY 1974 for the review and funding of competing research grants, fellowships, and training grant awards.

In mid FY 1973, when the fellowship and training grant programs were terminated, there were 46 Research Career Development Award Applications remained on hand; 24 of these applications were reviewed in FY 1973 and 22 were

reviewed in the spring of 1974 for award of released impounded funds. Included in this group were the Allergic Diseases Academic Awards of which 5 had been approved for funding. With the release of impounded funds, we were able to fund 4 awards in the NIAID Allergic Diseases Academic Award program and 23 Research Career Development Awards. All but one of these applications were funded for the full 5-year period. During FY 1975 both the Research Career Development Award and Allergic Diseases Academic Award programs will be reinstituted. These will be valuable program instruments for us during FY 1975.

Allergy and Immunology Research Committee

The Allergy and Immunology Research Committee was established to provide advice and expertise to the Department in the fields of allergy, immunology, and immunology of infectious diseases in their primary review of applications for special clinical research programs, fellowships, and special development award programs.

The Committee has met 4 times for consideration of applications for payment in FY 1974. These included applications received in FY 1973 but paid in FY 1974 with FY 1973 released impounded funds. Thus the Committee provided scientific primary review for the following applications: 11 Asthma and Allergic Disease Centers, 4 Allergic Diseases Academic Awards, 5 Program Projects, 18 Research Career Development Awards, and 40 Institutional Research Fellowships. The Institutional Research Fellowships are to be brought to June 1974 Council, although it is anticipated that a small number of these will be paid in FY 1974.

Staff Manpower Study

The staff manpower study started under Departmental direction in FY 1972-1973 was continued, and data was gathered to compare FY 1974 with FY 1973. Because of many program changes and because of the action of released impounded funds causing a tremendous shift to the activities concerned with awards and review of applications in the last half of 1973, it is unclear whether 1974 can be compared with 1973. The exercise was run in order to further establish the feasibility of this scheme for allocating positions among Extramural Programs.

Program Evaluation

The pilot study of a system of evaluating programs which was undertaken in FY 1973 was continued at a low level in FY 1974. There has been considerable interest in this activity by those on NIH/OD staff interested in program evaluation. The effects of previous evaluations on staff and Council ability to make decisions has been most rewarding. The idea of providing grantees within these programs with feedback from the evaluation exercise also appears to be valuable. Several more programs will be evaluated during FY 1975.

Budget

During the past fiscal year a change was made in the general format for presentation of the President's Budget. In preparing the FY 1975 Budget, requests for funds were made by program areas rather than by mechanisms. Budget "crosswalks" were developed to show not only the mechanisms involved but also the dollar requirements of the entire Institute in each of the four program areas: Allergic and Immunologic Diseases, Bacterial and Fungal Diseases, Viral Diseases, and Parasitic Diseases. Fiscal reports were changed to make current data "comparable" with traditional reports and historical data "comparable" with the new program budget concept. Formats for machine reports were to be redesigned to reflect activity within each of the four programs as well as recording the mechanisms involved. A new account management structure system was proposed which will necessitate a complete change in accounting procedures within the Extramural Programs. As a result of this change each award and application, active or pending, in research, fellowships, and training activities must undergo a rereview by staff for identification by program with the resulting information fed into our data control system. Activities, which in the past have remained fairly stable, have been phased-out, reactivated, restructured, and reshuffled within budget categories during the past 12 to 16 months.

Since the FY 1973 released impounded funds were actually awarded during the course of FY 1974, the numbers of these awards and the dollar amounts involved have been included in the totals reported for each individual program review appearing in the sections which follow.

Personnel

During the past year, we have been fortunate in having an experienced staff who has been able to adapt to new programs and changes in procedures. Their experience and versatility has allowed them to shift to areas where they were needed most and provide uninterrupted efficiency under sometimes difficult circumstances.

Dr. Roman Kulwich, who had served as Assistant to the Associate Director for Program Review and Evaluation as well as Chief of the Review and Evaluation Branch, was appointed as Assistant Associate Director with the accompanying involvement in the planning and direction of the total Extramural Programs. He has also continued to function as Chief of the Review and Evaluation Branch.

Early in the fiscal year Mr. Frederick Norton, who had served as Chief of the Grants Management and Operations Branch since May 4, 1969, transferred to another part of the Department. Mrs. Eleanor Wyatt, former Head of the Grants Management Section, has replaced Mr. Norton as Chief of the Branch. Mrs. Grace Ellis, serving under the direction of Dr. Roman Kulwich, provides the budget and fiscal information previously maintained by Mr. Norton.

The Grants Management and Operations Branch has been reorganized in order to provide a more streamlined supervisory structure. The organizational changes also allow the management and processing staff to work more closely in their assigned scientific program areas.

The administrative support staff has been established as a section within the Office of the Associate Director. Thus, it can directly serve the branches and interact with counterparts in other segments of the Institute and the NIH.

Four members of the EP staff have received quality increases for their sustained superior work performance. They are Mrs. Mattie Tynan, Mrs. Rosalie Strauss, Miss Linda Tozier, and Mrs. Evelyn Rosenstein. It has been through this type of dedication on the part of our staff that we have been able to handle the increased work load with the decrease in number of employees. (See Table II.)

We have attempted to develop the staff by providing formal training for 22 members (46%) of the Extramural Programs' staff who attended 40 courses at a cost of over $1700. The cost did not include the salaries while training, nor did it include the support of the STEP courses or Upward Mobility Program.

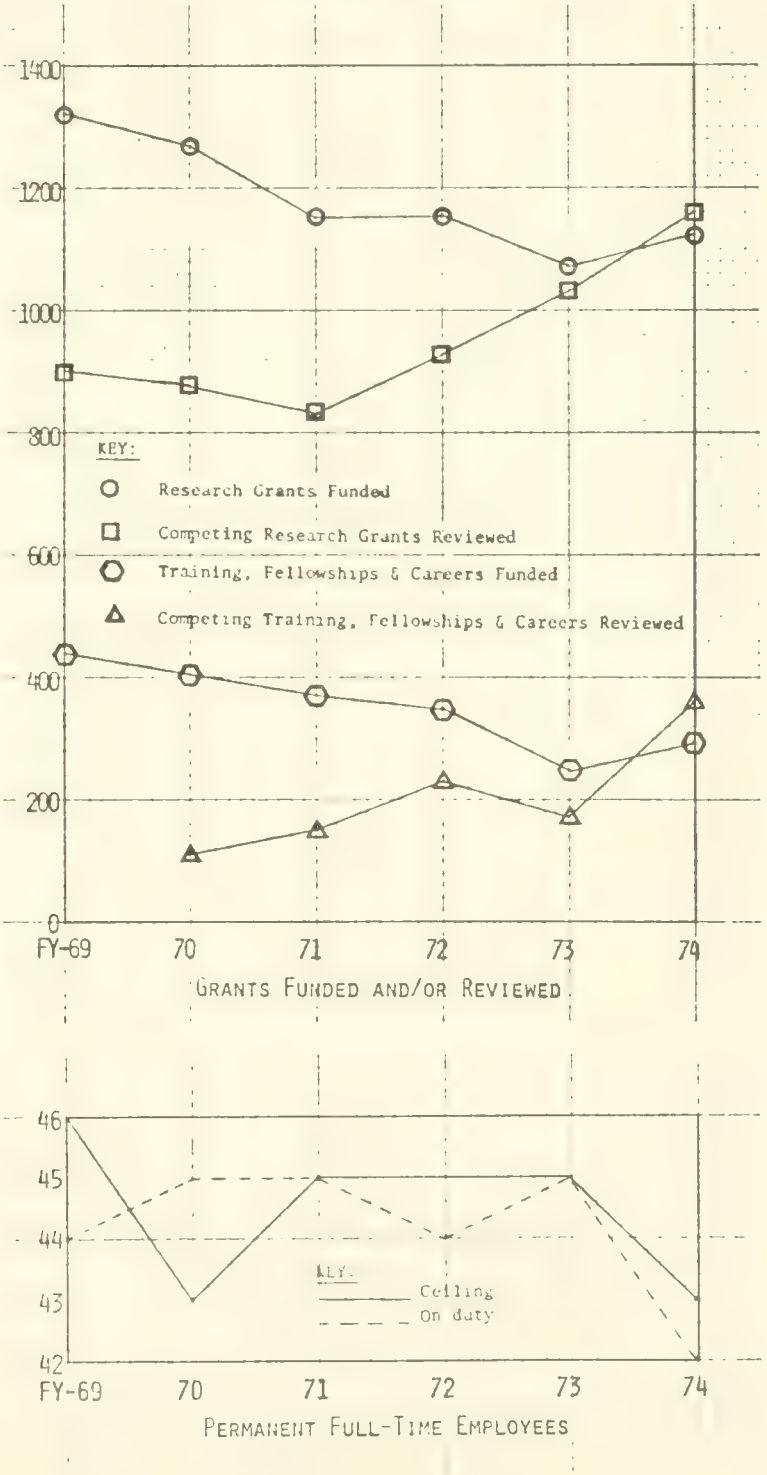
TABLE I

NIAID Competing Research Grants, Fellowships, and Training Grant Awards Reviewed, Approved and Funded

Category	FY 1973 Requests			FY 1974 Requests
	Regular Funds	Released Impounded FY 73/74 Funds	Total Outcome	
Research Grants				
Reviewed	-	-	990	977
Approved	-	-	745	788
Funded	283	52	335	300
Percent of Approvals Funded	-	-	45	38
Restoration Supplements Funded	-	80	-	-
Research Career Program				
Reviewed	40	22	62	0
Approved	25	15	40	0
Funded	4	27	31	0
Fellowships (Old Program)				
Reviewed	-	-	134	-
Approved	-	-	116	-
Funded	14	14	28	-
New Research Fellowships				
Individual				
Reviewed	-	-	-	335
Approved	-	-	-	290
Funded	-	-	-	86
Institutional				
Reviewed	-	-	-	39
Approved	-	-	-	25
Funded	-	-	-	5
Training Grants (Old Program)				
Reviewed	-	-	52	0
Approved	-	-	47	0
Funded	3	16	19	0
Restoration Supplements Funded	-	95	-	0

TABLE II

NIAID Grants Funded and/or Reviewed — Manpower
FY-1969 thru FY-1974



II. PROGRAM REVIEWS

A. ALLERGY AND IMMUNOLOGY

The approximate level of support given allergy and immunology:

Type of Grant	Number	Amount
Research grants	320	$20,772,378
Training grants	43	1,736,478
Fellowships	45	654,730(1)
Research Career Development Awards	36	828,815
Allergic Disease Academic Awards	5	137,000

Summary

Progress of research in the basic mechanisms of immunologic phenomena continues to involve relevant areas of biochemistry, cell biology and genetics. With these expanding contributions in immunobiology, immunochemistry and immunogenetics has come increasing insights into pertinent mechanisms of human disease. Continuing efforts to understand host defense at both cellular and humoral levels have been responsible for bringing in meaningful information on the mechanisms and pathways of immunity in health and their corresponding deviations in disease. Thus, researching the factors for induction, regulation and manipulation of the immune response is finding relevance and applicability to control of infection by bacterial, viral, fungal and parasitic organisms, problems of transplantation, autoimmune and allergic processes, tumor growth and rejection, immunodeficiencies and aging.

Some of these research areas responsible for especially important contributions and advances are:

Transfer factor - The ability to extract a soluble material from blood lymphocyte cells capable of transferring specific types of the donor's immunity to a non-immune recipient has been an interesting experimental procedure for several years. A real advance in the large scale preparation and purification of this substance known as "transfer factor" allowed for its application to human disease. Its potential therapeutic efficacy has been demonstrated in the study of a spectrum of infectious processes based upon the affected patients' cellular immunologic deficiency, e.g. leprosy, oromucocutaneous candidiasis, coccidiomycosis, Wiscott-Aldrich syndrome. Significant progress has also been made in uncovering and defining the exact chemical character of "transfer factor" to facilitate understanding its mode of action and possibly extending its use of the treatment of cancerous processes associated with cellular immunodeficiency, e.g. Hodgkins Disease.

Lymphocyte Biology - Specific cells involved in immune mechanisms and functions have been identified. From animal studies it was found that

(1) Includes 3 Institutional Fellowships.

there are two distinct types of lymphocytes responsible for the induction and maintenance of the immune process, T cells of thymus origin and B cells derived from bone marrow. Researching and establishing techniques to identify and separate T and B cells in humans by utilizing research knowledge of their surface membrane chemistry has advanced our ability to define their presence and functional states in health and disease. We are now learning that specific B or T cell disorders are characteristic of diseases of unknown etiology, e.g. acute and chronic lymphocytic leukemias, mycosis fungoides, autoimmune disorders, which in turn should offer significant leads for the treatment of these often fatal diseases.

Immunogenetics - The importance of genetic factors has been consistently demonstrated in the area of transplantation compatability where there is primary need to demonstrate similarities and/or differences between donor and recipient. It has now been found that genetic factors are equally important in determining and regulating serum antibody mediated immunity. Consequently, we are learning that one's hereditary makeup by specific immune response (Ir) genes can be responsible and influence immune factors in functions of maintaining health and in the induction of human disease.

Chemical mediators of immunity - Our knowledge of the mechanisms responsible for inflammation and allergy as undesirable side effects of immune reactions and the physiologic pathways of resultant protection against and induction of human diseases has been advanced by precise chemical studies. The chemical and biologic characterizations of compounds generated and/or liberated during immune reactions (histamine, kinins, complement fragments, SRS-A) are enabling clinical immunologists to understand and in turn seek correspondingly effective methods for treatment of asthma, anaphylactic shock, tissue inflammation and lack of immunity resulting from defective cellular engulfment and destruction of bacteria. The study of intracellular enzymes have helped to explain the role of cyclic AMP levels in determining whether immunity or hypersensitivity may ensue. This has led to the development of a new field, immunopharmacology, which shows promise for enhancing and controlling immune reactivity by the use of pharmacologic agents in immune-related and allergic diseases. Other significant advances in the field of immunochemistry involves the function of antibody immunoglobulins, e.g., the site of action of IgE on cell surfaces in the membranes of allergic patients, the site for effective action of IgA in preventing virus and bacterial invasion at respiratory, urinary, and gastrointestinal tissue sites, and the defining of the complicated structure of IgM, the blood borne antibody responsible for the development and maintenance of immunity against bacterial invasion.

Relationships between immunodeficiency, autoimmune and neoplastic diseases - Studies applying newly gathered information on immune processes and reactions are demonstrating that human diseases of immunodeficiency, autoimmunity and tumor growth may not fall into totally distinct categories. As we learn more about their inter-related basic etiologic and pathologic factors, leads for prevention and control are being investigated. It is becoming increasingly evident that cytotoxic drugs used in attempts to destroy tumors have serious, uncontrolled and deleterious side effects by their concomitant destruction of immunocompetent cells. Hope for control of tumor growth lies in the cor-

rection of associated immunodeficiencies and immune disorders to allow for the rejection of cancerous processes. Where serum antibody protects the tumor from attack by body lymphocytes, means have been designed to enhance the efficacy or restore defective cellular immunity. Much of what we know about the body's defenses and sensitivity reactions is being applied to effective manipulations for rejection of tumors, retention of transplanted organs and tissues, recognition of self tissue to prevent the development of autoimmune diseases (sensitivity against one's own cellular proteins), and the reconstitution of natural or induced immune deficiences by bone marrow and thymus transplants. We are learning how failure of the body to eradicate viruses may lead to overproduction of antibody resulting in injury to tissue (e.g., kidney glomerulonephritis) by the continued presence of antigen-antibody and products of their interaction.

Meetings

1. Asthma and Allergic Disease Centers Workshop: The Third Annual meeting for program directors of the 14 Centers and members of their staffs, organized and sponsored by NIAID, was held in Bethesda on December 6-7, 1973. Dr. John H. Vaughan, Director of the AAD Center at Scripps Clinic and Research Foundation in La Jolla, California served as Chairman. Presentations and topics devoted to research in asthma discussed included: multidimensional classification of asthma, inhalational challenge studies, beta blockade and cyclic nucleotides, immunoglobulins and chemical mediators, aspirin sensitivity, role of infection, idiopathic asthma and hypersensitivity pneumonitis.

2. Bronchial Inhalation Challenge Workshop: The six Asthma and Allergic Disease Centers conducting clinical investigations based on bronchial inhalational challenge techniques have formed a committee for cooperative studies. Two workshops for collaborating investigators under the Chairmanship of Dr. Richard S. Farr, Director of the AAD Center at National Jewish Hospital in Denver, Colorado were held at Chicago, Illinois May 31-June 1 and at Bethesda, Maryland, December 4-5, 1973. This group is currently concerned with standardization of instrumentation, techniques and reagents.

3. Clinical Immunology Workshop: The NIAID-EP has cooperated with the American Association of Immunologists Committee on Clinical Immunology working to define the scope and definition of clinical immunology as a research and clinical discipline of medicine and the optimum function and organization of clinical immunology units. NIAID-EP participation and partial support was given to a workshop-type meeting of this group, under the Chairmanship of Dr. Kurt J. Bloch, Director of the Arthritis Unit at Massachusetts General Hospital, Harvard Medical School, February 8-9, 1974.

4. Immunological Tolerance, Mechanisms and Potential Therapeutic Applications: An NIAID-EP sponsored and supported workshop on immunological tolerance under the Chairmanship of Dr. Baruj Benacerraf, Professor of Pathology at Harvard Medical School, was conducted at Brook Lodge, Michigan on April 28-May 1, 1974. Thirty-four of the foremost scientists with interests and programs in this research area from the U.S. and foreign countries attended and participated in this endeavor. Major subject areas discussed included T cell

tolerance, suppression and immunity, B cell tolerance, induction and mechanisms, T and B cell interactions, suppressive activity of cells, antibody and immune complexes, and potential therapeutic applications.

Continued productivity especial relevance to the functions of this Institute and the desirability of meeting research needs in selected areas have justified continued existance and support of Special Emphasis Programs.

1. Asthma and Allergic Disease Centers
2. Clinical Immunology and Immunopathology
3. Lymphocyte Biology
4. Selective Activation of Cell Mediated vs. Humoral Immunity

Asthma and Allergic Disease Centers Program

The addition of seven new Centers at Mayo Clinic, and Northwestern, Louisiana State, California-San Francisco, Duke, Colorado and Creighton Universities bring the total of AAD Centers to 17. The objective of this program is to support and encourage the development of interdisciplinary groups posessing the capability of translating their accomplishments and productivity in laboratory-based immunologic research to clinical investigations in hypersensitivity states and allergic diseases of man. Subjects especially earmarked for study include etiologic factors of asthma, hypersensitivity pneumonitis, immunotherapy, roles of infection, heredity and immune deviations in the development of allergy, modulation of factors governing the natural history of atopic states, chemical mediators, intracellular enzymes, immunopharmacologic agents and neuroreceptors in the expression of asthmatic and eczematous processes, and the development of new and precise diagnostic laboratory procedures and specific therapeutic approaches.

Program Highlights

AI-10356-03 - K. F. Austen

The immunologic release of clinical mediators of immediate hypersensitivity in human lung tissue and nasal polyps have been demonstrated to come under both adrenergic and cholinergic control. IgE deposition has been identified in involved kidneys of some patients suffering from systemic lupus erythematosus. In systemic lupus erythematosus the classic complement pathway is activated with recruitment of the alternate pathway during exacerbations of the renal disease; whereas in hypocomlementemic chronic glomerulonephritis activation of the alternate pathway is selective.

AI-10398-03 - R. S. Farr

Basophils in the peripheral blood behave less specifically than mast cells in the degranulation-histamine release process, and may not be significantly correlated with sensitivity of skin, bronchi and reaginic content of serum, and thus furnish an unreliable index of hypersensitivity and results of immunotherapy. Bronchial inhalational challenge studies indicate that it

may be possible to understand asthma on a pathophysiologic basis of the size airways undergoing constriction. In the study of sinobronchial disease with asthma, naturally occurring immunoglobulins with the capacity to specifically bind antigens associated with bacteria and fungi have been identified.

AI-04646-12 - C. W. Parker

Studies on cellular enzymes indicate that cAMP may be important in early phases of mitogenic stimulation and that different adenylate cyclase stimulating agents act on specialized adenylate cyclases in different parts of the cell. Immunological approaches to the handling of drug resistant infections are encouraging. Antibody-enzyme conjugates directed to specific organisms have been developed as an adaptable mechanism for the treatment of patients with leukocyte dysfunction, fulminant bacteremia and refractory parasitic infections in man.

AI-10397-02 - C. E. Arbesman

In the study of allergic reactions to bee (hymenoptera) stings, it has been demonstrated that the venom contains a specific antigen not present in significantly detectable amounts in whole body extracts, phospholipase A (PLA) can be identified as a major antigenic component, protection against anaphylaxis can be achieved by injection treatments with venom but not whole body extracts, RAST using venom or PLA serves as an excellent laboratory tool for measuring hypersensitivity, and that the reaction to each species of the stinging insects may be specific.

AI-10304-03 - P. S. Norman

In the study of patients with asthma due to ragweed pollen utilizing leukocyte histamine release and bronchial inhalational challenge tests it has been demonstrated that lung and skin mast cells and circulating basophils respond as a single population of cells, and the diagnostic valve of quantitative skin test procedures has been established.

AI-10404-03 - C. E. Reed

In the study of asthma induced by infectious agents only the rhinoviruses appear to be etiologically important for engendering the triggering inflammatory stimulus. Susceptibility related to infectivity has been found to depend upon a quantitative factor, i.e., the number of viral particles shed and transmitted by direct contact.

Clinical Immunology and Immunopathology

This program is concerned with the causes, diagnoses, distinctive pathology and treatment of transmitted and acquired immunologic derangements and abnormalities. An important objective is the translation of experimental studies to clinically related investigations of human diseases arising on an immune basis, e.g., immunodeficiencies, autoimmune reactions, hypersensitivity phenomena and failure of immune responsiveness.

AI-11313-02 - H. O. McDevitt

Clinical correlations have been established between the presence of extremely high titers of antibody to ribonucleoprotein (RNP) and the mixed connective tissue disease syndrome in which kidney involvement and autoantibodies to deroxyribonucleic acid (DNA) are infrequent. Subacute systemic lupus erythematosus with nephritis has been found less severe when antibody to Sm antigen is present along with DNA antibody. It has been shown that Sm antigen binds DNA, thus preventing its reaction with anti DNA. In the search for direct correlation between histocompatibility type, immune response and susceptibility to disease, patients with thyroiditis have been studied. It appears that HL-A8 antigen serves as a genetic marker for propensity to develop autoantibodies to thyroid microsomal antigens in association with Graves disease; whereas the HL-45 antigen serves as a genetic marker for propensity to develop autoantibodies to the same antigen but without any attendant clinical illness. They may represent different immune response genes responsible for the development of antithyroid microsome autoantibodies with different degrees of cellular autoimmunity or of antibody mediated thyroid damage. Though a majority of patients with nodular sclerosing Hodgkin's Disease have normal concentrations of peripheral blood T and B cells, a qualitative defect in function of T cells or alteration in T cell subpopulations leads to diminished responsiveness in tests of cell mediated immunity. In studies with Herpes simplex virus deficiency of T cells depressed the ability of lymphocytes to respond to viral antigen at least 20-fold indicating that it is the T cell responsible for the production of interferon.

AI-07007-09 - F. J. Dixon

In animal models a C type RNA virus isolated was found capable of producing antinuclear antibodies, associated with lupus nephritis and lymphoma-leukemia. Advances were made in the study of antiglomerulo-basement membrane glomerulonephritis in isolating an human nephritogenetic GBM antigen-antibody reaction. Susceptibility to chronic lymphocytic choriomeningitis (LCM) infection may be genetically determined and with immune responsiveness controlled by Ir genes; LCM virus levels in chronic infection determined by non-immune mechanisms.

W. O. Weigle

Methods of bypassing the need for T lymphocytes in the immune response and ways of inhibiting the induction of immunological unresponsiveness at the B cell level while maintaining T cell unresponsiveness have been demonstrated. Specificity of T cell unresponsiveness as well as the nature of a specific T cell marker has been defined.

G. C. Cochrane

In the study of allergic reactions and the mechanisms of deposition of injury effecting immune complexes in tissue a cooperative relationship between release from tissue mast cells and platelet activation upon stimulation with

antigen has been identified. In an animal tumor model it was shown that murine sarcoma cells grow exclusively by proliferation thus facilitating the assessment of the role of regression of virus antibody.

H. J. Muller-Eberhard

Discovery of the nephritic factor often found in patients with hyprocomplementemic nephritis was shown to be distinct from immunoglobulins and a potent activator of the alternate pathway.

M. B. A. Oldstone

It was found that in addition to renal glomeruli and arteries, the choroid plexus is a favored site for deposits of antigen-antibody complexes, thus helping to clarify the behavorial disturbances and disorders of consciousness observed in patients with systemic lupus erythematosus.

V. A. Bokisch

Increased consumption of complement has been demonstrated during Dengue hemorrhagic fever pointing to its patholgenetic importance in the development of the hemorrhagic shock syndrome.

AI-05877-11 - F. S. Rosen

Considerable progress has been made in defining specific complement component defects to patterns of immunodeficiency diseases on a genetic basis. In the case of agammaglobulinemia specific B cell abnormalities were identified in the hereditary x-linked disease. In addition to B cell deficiencies, in vitro techniques point out unresponsiveness of agammaglobulinemic B cells in some situations; a number of hitherto undetected immunodeficiency states have been described in children, e.g., IgG subclass deficiencies with pyogenic infection, absence of circulating mature T cells in thymic aplasia with cardiovascular anomalies. Success has been achieved in bone marrow transplantation in children with aplastic anemia and bone marrow aplasia with determinants at a locus other than the major histocompatibility one having been found a critical factor. Since a major cause of morbidity among immunodeficient patients is infection due to Hemophilus influenza, type b, the identification of a purified capsular component, polyribose phosphate, as the immunogenic factor may lead to the feasibility of protective immunization.

AI-05754-11 - J. E. Salvaggio

The findings of a significantly greater rise in nasal secretion anti-tetanus toxoid antibody titers resulting from intranasal immunization suggest that the atopic subject might produce functionally defective nasal secretory IgA antibody although exhibiting normal serum and secretory total levels. In the study of immunologic mechanisms involved in hypersensitivity pneumonitis culture of most environmental isolates were

Thermoactinomyces vulgaris based on amino acid cell analyses and there appeared to be a lack of shared "cellular" antigens between these and Micropolyspora faeni, the richest source of "farmer's lung hay" antigen.

AI-10704-14 - A. F. Michael

In studies on steroid resistant idiopathic nephrotic syndrome in man, it was possible to show that recurrence of the disease occurs in the transplanted kidney suggesting that non-renal factors are operative; whereas no recurrence occurs in congenital nephrotic syndrome after transplantation. Alterations in the alternate complement system involving properdin and C3 proactivator have been identified in the pathogenesis of some immune mediated renal diseases, e.g., lupus, chronic membranoproliferative and acute-post streptococcal glomerulonephritis. An increased uptake of macromolecular aggregated IgG was demonstated in nephrotoxic nephritis suggesting an important relationship between mesangial function and glomerular filtration. In pemphigus, significance of a suspected autoimmune component has been added by the demonstration of decrease in blister fluid content of complement components.

Lymphocyte Biology

Lymphocytes, cellular elements populating bone marrow, thymus, lymph nodes, spleen, blood and lymphatic fluid are critical to the immune system by their diversity of participation in highly specialized biologic functions. Individually specific types of lymphocytes are each responsible for recognition of a foreign molecule at its cell surface, transmissal of informational signals to other cells characterized by immunological competence, and production and secretion of specific types of antibody molecules, i.e., immunoglobulins, retention of immunologic memory or the production and release of chemical mediators having biologic activity. The Lymphocyte Biology Program was developed to attain complete knowledge of the life history of immunocompetent cells and the genetic factors that determine their fate and function. Such knowledge is of great importance in providing information to effectively reconstitute immunodeficient patients, alleviate allergic states, resist virulent infections and to combat neoplastic processes.

To further the advancement of such an approach, multidisciplinary programs have been accomplished through NIAID research grants to effectively integrate the techniques of cell culture, cell biology, genetics, virology, immunochemistry, and cellular immunology. The addition of two program projects, at University of Texas, Southwestern Medical College and the University of Wisconsin brings the total of research centers participating in NIAID's Lymphocyte Biology Program to four.

Program Highlights

AI-11378-01 - G. M. Edelman

Development of the fiber-fractionation technique adds an effective method for specific collections of functioning T and B cells. Analysis of dis-

tribution and mobility of immunoglobulin receptors on B cells has provided important clues to factors controlling receptor mobility. Antigens that are serologically non-cross reacting do cross react at the cellular level indicating that both specificity and affinity play central roles in the antigenic stimulation of lymphoid cells. A subpopulation of T cells does not appear until two-three weeks after birth, correlating this population with the appearance of an immune response.

AI-08439-07 - F. H. Bach

In an animal model of human histocompatibility, it has been established that reactivity of lymphocytes is controlled by loci other than those controlling serologically defined (SD) antigens of H-2K and H-2D; these loci have been mapped out. In vivo studies of histocompatibility applied to the pathogenesis of graft vs. host reaction (GvH) and secondary disease have demonstrated that GvH are determined primarily by LD locus differences of H-2 rather than SD differences. LD-2 loci differences are associated with skin graft rejection without known H-2K or H-2D disparity; LD-1 differences permit long graft survival. Methods have been developed for the production of antisera against mixed leukocyte culture (MLC) - important gene products; blocking recognition of value for in vivo manipulation of the in vivo response.

AI-11851-01 - J. W. Uhr

Ig+ cells, probably plasma cells and/or bone marrow-derived (B) lymphocytes of non-thymocyte lineage have been demonstrated within the thymus. Studies on the turnover and release of cell surface Ig and secretion of total intracellular Ig indicate that: binding of macromolecules (Ig) to the interior of Golgi vesicles is a critical step in the transport of cell surface proteins, later released and secreted. In contrast H-2 alloantigens are integral glycoproteins of the plasma membrane and may be part of the post-Golgi vesicles which transport Ig to the plasma membrane. A model system studying the binding of antigen to cells with antibody on their surface with respect to the energetics of interaction suggest definition of the mechanism by which multivalent antigen can stimulate immune cells in the presence of circulating antibody.

Cell-Mediated Versus Humoral Antibody Responses

This program seeks to understand interrelationships between cell-mediated immunity and humoral antibody formations at the interface of their respective generation, regulation and expression. The importance of this possible capability to selectively manipulate the pathway of the immune response could be applied to the prevention and treatment of infectious diseases and neoplasias and the control of adverse transplantation reactions and aging.

AI-01254-18 - H. S. Lawrence

Perfection of an in vitro assay of transfer factor (TF) by its ability to convert non-immune blood lymphocytes to immune reactivity is considered a break-through for research designed to elucidate the molecular identity and mechanism of action of the active moiety in TF.

AI-09920-04 - B. Benacerraf

Studies concerned with the activity of histocompatibility-linked immune response genes (Ir) indicate that the Ir gene is involved in carrier recognition and suggest that it operates in thymus-derived lymphocytes. Studies on the cooperative interaction of T and B cells in humoral immune responses indicate that x-irradiation does not result in abrogation of T cell helper function and production of soluble inhibitory mediators in antigen-induced suppression (antigenic competition); T lymphocytes elaborating such mediators. The affinity of receptors on precursor cells for tolerogen appear to be crucial determinents of whether an individual cell will become tolerant.

AI-10904-02 - H. J. Winn

An alloantibody to B cells has been defined in an animal model by genetic, cell population and functional studies which in turn may permit greater manipulation of the immune response than is currently possible. Products of H-2 loci have been found to differ in both respective humoral and cellular immunogenicity in graft rejection and enhancement. The finding that peripheral coating of the graft is not the likely mechanism of tumor enhancement appears also applicable to tumors indicating a primary central rather than a peripheral role in prolonged tumor growth.

AI-11041-02 - T. L. Feldbush

The existance of two memory cell populations of different half lives have been demonstrated indicating the necessity of continual antigenic stimulation in order to perpetuate immunologic memory; one concentration of antigen optimal for stimulating antibody formation; a lesser amount for potentiating memory. Determination of the anatomical distribution of short and long-lived memory cells indicating the same relative percentages among spleen cells and lymph node and thoracic duct lymphocytes requires re-examination of existing information on the biologic life span of thoracic duct lymphocytes.

AI-11077-01 - F. S. Kantor

The process of desensitization was shown to depend upon sensitization indicating that sensitized cells act by producing an inhibitor causing unresponsiveness to the indifferent antigen. In the study of anergy associated with sarcoid, decrease in functional capacity of T lymphocytes related to steroid treatment. Hapten specificity of transfer of cutaneous basophil hypersensitivity has been defined helping to clarify the separation of this form of delayed-in-time reaction from the conventional delayed hypersensitivity. The immunologic defect in sarcoidosis is not due to dysfunction or depletion of lymphocytes, but that the anergic phase may be caused by an immunosuppressive factor.

AI-10886-02 - J. J. Marchalonis

The presence of immunoglobulin comprised of light and μ chains was found on the surfaces of all normal lymphocyte populations. T cell immunoglobulin did not bind to either T or B cells, thus militating against a cytophilic origin. Direct evidence was found for surface immunoglobulin of activated T cells functioning as an antigen receptor.

Other Program Highlights

Immunochemistry

AI-09810-04 - J. D. Capra

Amino acid sequence of Ra5, an allergenic component of ragweed pollen has been characterized.

Immunogenetics

AI-08897-06 - D. B. Amos

In studies of tolerance to heterologous immunoglobulin systems, it was shown that induction and maintainence of tolerance involved neither blocking factors nor suppressor cells. Carrier-specific T cell tolerance was shown to be long-lasting and dose-dependent. Cross-tolerance attributed to T cell cross-reactivitiy was demonstrated.

Transplantation Immunology

AI-10678-02 - R. E. Billingham

Decidual tissue conferred no protection upon intra-uterine allografts suggesting that it interferes only with the afferent pathways of the uterine reflex. Allogenic sperm and fetuses incite lymphadenopathy but not transplantation immunity. Evidence was obtained for fetal vs maternal reactivity attributable to fetal immunocytes. There is thus no reason to believe that feasible conception control can be based upon transplantation alloantigens. Maternal and fetal immunogenetic disparity affect placental size and this manipulation also affects trophoblastic invasion and proliferation. Viable milk-borne lymphocytes are observed in appreciable numbers in neonate tissue. Though the anterior chamber (AC) of the eye is an immunologically priviledged site, graft size and associated non-specific inflammation can abrogate the protection afforded by this segment of the eye. Even in immunologically naive hosts, antigen reactive cells can recognize antigens on lymphocytes within the AC and effect destruction of cells in the absence of lymphatic drainage.

AI-10060-04 and AI-11202-02 - K. Ishizaka

IgE and IgG enhancing antibody factors are different. T and B cells for ragweed antigen E have different specificities. Cells which cannot be sensitized by IgE fail to release histamine on antigen challenge because

they are poor responders to antigen-antibody surface reactions. Patch or cap formation due to IgE reaction is not a process of histamine release.

AI-10686-02 - L. Spitler

Transfer factor (TF) prepared in this laboratory has been used therapeutically to induce cellular immunity in international cooperative studies. Diseases in which TF was effective included Wiskott-Aldrich syndrome, severe combined immunodeficiency disease, mucocutaneous candidiasis, chronic active hepatitis, coccidiomycosis, dysgammaglobulinemia, Bechet's disease, apthous stomatitis and malignant melanoma. Since not all patients in each category responded to the administration of TF, different forms of the various diseases may have different pathogenetic mechanisms although appearing clinically similar.

AI-10088-04 - R. L. Walford

In the study of chronic lymphocytic leukemia, antigenic factors not related to the HL-A system have been demonstrated; there may be different antigens specific for different varieties or subdivisions of chronic lymphatic leukemia. However, in contrast to leukemia patients, no hyperactivity of HL-A typing sera were seen with cells from patients with lymphomas. Aberrant HL-A antigen distribution was found in a study of patients with myesthenia gravis, especially related to sex and age of onset.

B. BACTERIAL AND MYCOTIC DISEASES

The approximate level of research support in bacterial and mycotic diseases:

Type of Grant	Number	Amount
Research grants	242	$10,237,149(1)
Training grants	38	1,640,926(2)
Fellowships	22	347,870

Summary

The name of the Bacteriology and Mycology Branch was changed to Bacterial and Mycotic Diseases Branch, as this name more accurately reflects both the nature of the research programs it supports and its relationship to the National Institute of Allergy and Infectious Diseases. Research progress in the high-quality grant program supported by the Branch continues to uncover significant or noteworthy new results, many of them showing considerable future potential for direct clinical and therapeutic application.

The Special Emphasis programs of this Branch have continued their growth, indicating the very great interest of the biomedical science community in these programs. This is most notable in the Special Emphasis programs in Biology of Venereal Diseases and in Hospital-Associated Infections. Only the best of the many approved grants in these programs are selected for funding under the Dollar Allocation by Program Area (DAPA) system adopted in the past. The Special Emphasis program in Streptococcal Diseases and Sequelae was considered well enough developed by the National Advisory Allergy and Infectious Diseases Council that it did not require a special funding mechanism (DAPA) in 1974. The Special Emphasis program in Mechanisms of Drug Resistance was transferred to the Biochemistry and Physiology Branch for administration as this program, primarily one of basic research, was deemed more appropriate to that Branch. In its place a new Special Emphasis program in Hospital-Associated Infections was approved by the Council, with funding allocated under the DAPA system to initiate this program.

Other research areas covering the microbiology of important disease problems, outside the Special Emphasis programs, are continuing to show expanding interest by the scientific community. Research in fungal diseases and in infectious and diarrheal disease of the intestinal tract are some research areas fitting this category. Other disease areas of importance and interest are tuberculosis, staphylococcal infections, bacterial endotoxins, and leprosy.

(1) Includes 10 Career Awards.
(2) Includes 2 Institutional Fellowships.

Research Grant Highlights

A number of important research findings by grantees supported by the BM Branch are briefly summarized here:

Biology of Venereal Diseases

The greatest expansion has occurred in the Biology of Venereal Diseases program, which now comprises 34 research grants, one program project (a multi-faceted V.D. research center), a V.D. training grant, and several postdoctoral fellowships. Total investment currently is approximately $1,736,000.

AI-11305-02 - D. M. Musher

Greater clarity and definition of the immune response to syphilis are needed, particularly that related to cell-mediated immunity. Results of this study on lymphocyte stimulation of both normal and syphilitic patients indicate that lymphocytes from normal subjects were stimulated by protein extracts from a cultivable Treponema refringens. Patients with primary and secondary syphilis had a significantly-reduced lymphocyte response to the treponemal extracts. No effect was seen of normal serum on lymphocytes from the syphilitic patients and vice versa. These data support the hypothesis that specific immunologic suppression is part of the host response to infection with T. pallidum.

AI-11413-02 - L. J. Zubrzycki

The problem of increasing drug resistance of the gonococcus and the concomitant problem of treatment failures are becoming very worrisome. Results of this study have confirmed that many gonococcal strains resistant to one antibiotic are resistant to other, chemically-unrelated antibiotics. The data also suggest that multiple drug-resistant gonococci now seen may be resistant to antibiotics not currently in routine use. The antimicrobial resistance can be transferred by the DNA, and preliminary data indicate that there is an interaction between genes accounting for the resistance to single antibiotics. The possibility is strong that we may be facing a continuing step-wise increase in gonococcal resistance to single and multiple antimicrobials; it is also suggested that antibiotic resistance can be transferred between strains of gonococci in vivo.

AI-10615-03 - E. C. Gotschlich

Purified preparations of pili, obtained from virulent gonococcal strains, were labeled with radioiodine and used in the development of a radioactive antigen binding assay, as reported previously. Results of this test showed that mean antibody levels of 90% of asymptomatic infected females were higher than in noninfected controls. The pili antibody levels rose to peak values about two weeks following infection and declined to near-normal values within six months of successful treatment.

Antibody to pili also was observed in experimentally-infected chimpanzees, rabbits, and guinea pigs. Analysis of results indicates that the number of false positive results in this test in the infected female is very low; in infected males the results are not so clear-cut. A reliable blood test for detection of asymptomatic gonorrhea in females would be of significant advantage in lowering the rate of spread of the disease.

AI-07436-08 - H. E. Morton

This investigator is attempting to develop a more sensitive and specific serologic test for gonorrhea. In this study he has been able to adsorb the gonococcal antibody onto the surface of trypsin-treated sheep erythrocytes. The gonococcal antibody-sensitized erythrocytes served as the basis for a very sensitive indirect hemagglutination test, using only small numbers of the gonococcal organism in suspension. This test has great potential over the usual direct culture methods for diagnosis of the infection; the causative agent can be detected in very small numbers from body secretions before symptoms are recognized or antibody appears in detectable amounts. This test can be especially useful for diagnosis of gonococcal arthritis.

AI-03082-15 - Z. A. McGee

The increasing rate of penicillin therapy failure for gonorrhea may be due to persistence of the L-phase variant of the gonococcus during penicillin treatment. The conditions for formation and stability of gonococcal L-phase variants were determined by this investigator from fresh clinical isolates. These L-phase gonococcal variants were allowed to revert to their parent forms; on re-exposure to penicillin they again underwent transition to the L-phase far more readily than did the original gonococcal organism. These findings have implications for the disease in man, especially after penicillin therapy for gonorrhea.

Streptococcal Diseases and Sequelae

The evaluation of the Special Emphasis program in Streptococcal Diseases and Sequelae was completed in April of 1973 and presented to the Council in June 1973. In summary, the evaluators found that considerable progress had been made, and that the program was of sufficient importance to maintain it as a Special Emphasis program, but without the addition of DAPA funds. Of the 24 grants then in the program, eight were considered no longer relevant to streptococcal disease and were returned to the regular grants program. The summary report was forwarded to all members of the evaluation panel and the grantees involved. Currently the program comprises 19 grants, funded at $1,193,710.

AI-09645-05 - E. M. Ayoub

Rheumatic valvular heart disease is still of concern to pediatricians; although the disease causes serious disability, it is still a preventable disease. In this continuing study, the investigator has found

that certain oligosaccharides in mitral valves of rheumatic fever patients cross-react with antibody to the streptococcal carbohydrate antigen. He has also found that high levels of antibody persist in patients with rheumatic valvular disease for two to three years after the initial attack. Patients with congenital mitral valve defects or with non-rheumatic valvular disease show normal levels of streptococcal A antibody. These data lend further support to the role of an immunological mechanism in the pathogenesis of rheumatic valvular disease.

AI-02874-15 - A. W. Bernheimer

This investigator, a Research Career Awardee of the Institute, has continued to make valuable contributions to our understanding of streptococcal diseases. He has isolated and characterized certain specific bacterial proteins, to define their capacity to cause biologic and biochemical changes leading to lesions in the patient. In this current study Dr. Bernheimer has disproved the claim by others that streptolysin O was the same as nicotinamide adenine dinucleotidase (NADase). Purified streptococcal NADase had no hemolytic activity, nor did purified streptolysin O have any NADase activity. In other studies, a purified beta-hemolytic protein, sphingomyelinase from Staphylococcus aureus, was found to have a very high hemolytic activity. The sensitivity of the erythrocytes to this hemolytic activity correlated with the sphingomyelin content of the erythrocytes. This extracellular S. aureus enzyme may be of importance in pathogenesis.

AI-04342-13 - E. N. Fox

The goal of this study, rapidly approaching fruition, has been the development of streptococcal M protein for use as a suitable vaccine. Currently, children and adults were immunized with the purified M protein by a spray technique via the intranasal route. Only antibodies of the IgG class were detected in the sera of the vaccinees. Secretory IgA antibody from nasal washes or saliva showed no anti-M protein activity. In lactating rabbits immunized with the purified M protein, it was found that serum and colostrum had type-specific antibody, but without bactericidal activity. Other workers have recently shown that M proteins allow the streptococci to adhere to epithelial cells; the type-specific antibody prevents this adherence. Investigation is continuing on the participation of these type-specific secretory immunoglobulins in colonization and infection of mucous membranes by the organism.

AI-05100-12 - R. Austrian

The role of alpha-hemolytic streptococci in the pathogenesis of chronic infections of the lower respiratory tract is not clearly defined. This study is attempting to clarify the problem by developing criteria for classifying the alpha- and beta-hemolytic streptocci of the respiratory tract. It might then be possible to determine whether this large group of organisms play a significant role as opportunistic pathogens. Several strains of streptocci recovered by trans-tracheal puncture from

patients were found to have certain attributes relating them to the pneumococci. Some cross-reactivity between certain polysaccharide capsular components was found, as well as reactivity to their teichoic acids.

AI-11161-03 - C. Panos

This investigator, in his study on streptococcal L-forms, found that these wall-less forms could be stabilized and yet retain their capability for growth in certain media. Most importantly, the L-forms were found to attach to heart cells in tissue culture, and to penetrate and damage the heart cells, leading to extensive necrosis. These significant findings are the first to show that an osmotically fragile L-form has been found capable of tissue destruction *in vitro*. The possible role of L-forms in the pathogenesis of disease now becomes more creditable.

Hospital-Associated Infections

The program in Hospital-Associated Infections is beginning its expansion, with a current listing of 23 grants (a number of these being ongoing projects from previous years), funded at approximately $725,000. There is considerable interest expressed in this program by clinically-oriented scientists.

AI-10892-02 - R. L. Baehner

A highly-reactive and unstable intermediate radical called superoxide was studied in relation to its possible role in bacterial killing in polymorphonuclear leukocytes (PMN). It is known that the superoxide radical is produced by normal PMN during phagocytosis. Abnormal PMN, in patients with chronic granulomatous disease, fail to produce superoxide during phagocytosis, and these have greatly diminished or absent bacterial killing powers. This may be one reason for the greater susceptibility of such patients to infections, particularly to hospital infections.

AI-03082-15 - Z. A. McGee

A better understanding of host-parasite interactions will lead to more effective control measures than will the availability of new antimicrobial agents. The increasing incidence of disseminated *Candida* infections is being studied by examination of host factors responsible for overcoming *Candida* fungemia. In this study it was found that liver was the major organ for clearance of this yeast when injected into animals peripherally. *Candida* was present in liver granulocytes or enmeshed in fibrin clots and in Kupfer cells. The reticuloendothelia (RES) system of liver as well as circulating PMN seem to be the major contributors to the host's efforts to rid itself of the fungus.

AI-00023-24 - L. D. Sabath

This investigator has been studying the clinical pharmacology of antibiotics, particularly in the debilitated patient. He has found that oral antibiotic excretion or retention patterns vary widely among patients -- there is no true norm for any given antibiotic. This is particularly important for the severely-debilitated or the burn patient. Such patients, including those with renal failure, absorbed large amounts of neomycin, leading to neomycin toxicity. With potentially toxic antimicrobials, therefore, very fine regulation of therapy will require frequent and careful measurement of drug levels.

The problem of endotoxin shock as a serious concomitant to infection with Gram-negative microorganisms is intimately connected with the problem of Hospital-Associated Infections. Endotoxemia plays a significant role in the pathogenesis of Gram-negative infection, although the adverse effects of endotoxin on the host are poorly-understood.

AI-09394-05 - R. J. Moon

Results of this study show that serotonin production (from the breakdown of tryptophan) is increased in endotoxin-poisoned mice. Previous information showing the effect of endotoxin on liver enzymes and metabolism of carbohydrates suggests that excess serotonin plays a critical role in the hyperreactivity of endotoxin-poisoned mice. The author points out that disruption of metabolic hemostasis in the liver of these animals cannot help but play a detrimental role in the host's ability to cope with the endotoxin stress.

AI-07257-08 - J. W. Shands, Jr.

Studies of liver metabolism after endotoxemia and correlation with organ blood flow should help define what occurs first, shock or altered metabolism. Clarification of the mechanism of action of the endotoxin (LPS) is needed. Results of this study show that hypoglycemia occurs frequently before shock; there is also evidence for increased vascular resistance in the liver, with decreased blood flow. The loss of gluconeogenesis occurs about two hours after LPS administration. LPS was found to be cytotoxic for macrophages; studies with the electron microscope showed these cells to contain LPS in phagosomes, with ruptured membranes evident.

Fungal Infections

Fungal infections, particularly by opportunistic fungi, are becoming an increasingly grave problem in patients with severe underlying disease or under immunosuppressive therapy.

AI-10547-03 - R. I. Lehrer

Characterization of host factors related to resistance to systemic fungal infections is needed. This investigator has found in lysosomes of neutrophils fungicidal components that could kill Candida yeasts.

These fungicidal components were inhibited by several types of poly-anionic compounds. Analysis of neutrophils of patients suffering from systemic mycoses showed evidence of a lysosomal defect.

AI-05022-11 - G. S. Bulmer

In this study it was found that even activated alveolar macrophages were unable to kill Cryptococcus neoformans. The yeast cells remained viable after phagocytosis, even though the activated macrophages readily killed bacterial cells. Saliva from immunologically-compromised patients showed a reduced in vitro fungicidal activity compared to normal patients; a lower level of salivary peroxidase was found. These patients have a reduced ability to kill this yeast, whereas normal individuals are not particularly susceptible. Viable C. neoformans cells injected into mouse muscle enlarged and readily produced capsular material; this capsular material inhibited phagocytosis by neutrophils.

AI-10622-03 - G. Medoff

Therapy of serious, systemic fungal infections, with few exceptions, has not been notably effective. A newer method, using combined anti-microbials, now looks very promising with great potential for improved therapy of fungal infections.

Results of this study show that Amphotericin B., effective against fungal infections, also showed stimulation of peritoneal macrophages, probably owing to the membrane-active properties of the antibiotic. The anti-biotic's membrane effect is based on its greater avidity for ergosterol found in fungal cell membranes than for cholesterol, the constituent of mammalian cell membranes. This drug in combination with rifampin showed a strong synergistic effect; rifampin inhibits all types of RNA synthesis in the presence of Amphotericin B. A strong, irreversible binding was found that was correlated with fungal cell killing.

Cholera, Leprosy, and Tuberculosis

Cholera

Considerable interest is evident in the enterotoxin produced by certain toxigenic strains of Escherichia coli. These strains cause a diarrheal disease, similar in some respects to cholera, but less severe in its effects.

AI-07625-08 - N. F. Pierce

Enterotoxin produced by E. coli occurs in two forms, a heat-stable and a heat-labile toxin. Neither of these toxins is inhibited by cholera-genoid, the natural form of toxoided (inactivated but immunogenic) cholera toxin. A different binding site is therefore suggested; a ganglioside component of the intestinal cell may be the binding site for cholera toxin, but it is inactive for E. coli toxin. The effect

of prostaglandin substances supports the concept that prostaglandins and cholera toxin produce diarrhea by a common effect on small bowel mucosal transport of Na+ and Cl-; the inhibition of NA+ entry to the cell occurs at the mucosal border.

AI-11416-02 - S. T. Donta

This investigator has developed a unusual in vitro assay for diarrheagenic enterotoxin that may play a significant role in studies on the pathogenesis of the disease and on development of effective vaccine materials. The assay is based on the use of adrenal cells in tissue culture; when exposed to enterotoxin the adrenal cells undergo maximal steroidogenesis and show morphologic changes in specific response to the toxin. Further development of this assay also shows the system to be very sensitive for the detection of minute amounts of specific antitoxic antibody in human sera.

Leprosy

An experimental animal model for study of the pathogenesis of leprosy has been the goal of leprosy research for many years. Recently an exciting model has been uncovered which will undoubtedly play a significant role in future studies of this baffling disease.

AI-11620-01 - C. H. Binford

In collaboration with Dr. Eleanor Storrs and other scientists at Gulf South Research Institute, this investigator has helped develop the usefulness of the nine-banded armadillo as a most promising animal model for study of leprosy. This animal, native to the southwestern United States, has been found to be naturally susceptible to severe infection with Mycobacterium leprae. The severe infection, unlike that in other animal models (rat and mouse) occurs after inoculation with human leprosy material in the absence of any immunosuppression. This model now opens the way for exciting studies on the pathogenesis of the disease, and on drug therapy or immunologic methods for treatment of patients.

Tuberculosis

Development of more effective and acceptable therapy for the treatment of human pulmonary tuberculosis is still required. Although the disease is considered to be under control, in the United States in 1972, 33,000 new active cases occurred; a certain per cent of treated cases may in time develop antibiotic-resistant strains of the tubercle bacillus. Shortening the treatment period necessary to effect bacillary sterility of the pulmonary lesions is a major goal of such research.

AI-09478-04 - L. H. Schmidt

In this project Dr. Schmidt has been using sub-adult rhesus monkeys infected with virulent tuberculosis by inhalation challenge. He has found that combination therapy with isoniazid and rifampin gave the best therapeutic results. The most effective were doses of these two drugs, at specific concentrations per Kg. of body weight, given daily for four months. This system appeared to be uniformly effective in sterilizing the lesions in lungs and regional lymph nodes. No renewal of disease activity was found in post-treatment follow-up studies. This regimen was strikingly superior to treatment with these drugs given singly, and could therefore have a significant impact on treatment of human patients.

Other Disease Areas

Urinary tract infections are one of the major causes of morbidity in a large segment of the general population, accounting for large economic losses through lost wages and hospitalization charges. There are still many puzzling aspects of this disease entity yet to be clarified, although new information is becoming available which may lead to a better understanding of this problem.

AI-10388-16 - J . P. Sanford

A distinction between bacterial infection of the bladder and of the kidney is important in determining the prognosis and vigor of pursuit of diagnostic studies and therapeutic measures. This study has resulted in a new technique which enables localization of the site of urinary tract infections by a non-invasive method. It was demonstrated that bacteria in urine of patients with upper tract infection (renal bacteriuria) were coated with immunoglobulin readily detectable by fluorescent antibody techniques. Bacteria in urine of patients with infections of the bladder were not coated with this immunoglobulin. The distinction between bladder and renal bacteriuria is thus readily diagnosed; the method appears feasible and safe for routine use in diagnosis of urine with significant bacteriuria.

AI-10668-03 - W. R. Fair

This interesting study is on a unique prostatic antibacterial factor (PAF) found in men, to determine its role in urinary tract infections. Significant amounts of PAF are found in seminal plasma of normal men with no history of urinary tract infection. A marked deficiency of the PAF occurs in seminal plasma of men with chronic bacterial prostatitis. It was also found that PAF from rat, human, and canine prostate glands was identical, so that PAF is not species-related. Attempts are now underway to purify and determine the chemical composition of PAF. It is known to be a low M.W. compound, containing a few amino acids attached to a strongly-cationic substance, possibly a metal. PAF appears to be a unique substance in the male prostate gland, and may possibly occur in the periurethral glands of the female as well. This

is indicated by significant antibacterial activity, as measured by PAF activity, in vaginal washings of about 80% of normal females.

AI-09366-05 - T. A. Stamey

Urinary tract infections in women are extremely common, affecting about 10-20% of all women at some time during their lives. This study is to determine the conditions for colonization of the vaginal vestibule and its relation to urinary tract infections. Results show that no change in IgA or IgG antibody levels can be correlated with the presence of bacteria or change in colonization. It was also established that local vaginal antibody was not a factor in determining the susceptibility to urinary tract infections. Vaginal pH is apparently a major factor in colonization by the more common O-serogroup of E. coli; the less common serogroups are readily killed by lower pH levels. The pH defect relates to colonization and the local vaginal antibody types are not significant factors in the urinary infection.

Otitis

Otitis media is still a very common childhood disease. It is known that about 30% of children under five years have at least one episode yearly; by six and one-half years, 90% of children have experienced this.

AI-10838-02 - V. M. Howie

This study is to determine the secretory and humoral response to otitis media caused by Diplococcus pneumoniae or Hemophilus influenzae. The use of purified capsular polysaccharides from these two microorganisms, which cause otitis media, are presently under consideration as candidate vaccines. It becomes important, therefore, to understand the type and duration of the immune response to natural localized infections caused by these bacteria. It was demonstrated that seven types of pneumococcus are responsible for about 70% of pneumococcal otitis. Specific antibody occurs in acute serum and middle ear fluid (MEF). Specific antibody in MEF exclusively is found in about 60% of patients; this antibody is identified as the IgM type. Anti-pneumococcal antibody of the IgE class was also found in acute phase sera of patients. These data suggest that the secretory immune system may be activated locally to produce the specific antibody. A number of patients with specific antibody to MEF showed spontaneous clearing of their infection, suggesting an immunologic basis for the host management of pneumococcal otitis media.

Vaccine Development Studies

AI-11094-02 - R. P. Tewari

Study on the immunogenic properties of ribosomal RNA from Histoplasma capsulatum are being conducted in efforts to develop a safe and effective vaccine. The results show that purified ribosomal preparations with added adjuvant protected 70-90% of immunized mice; animals surviving challenge

also showed the absence of lung lesions and had negative spleen cultures. This protection reached a peak about three or four weeks post-immunization. RNAase treatment partly weakened the ribosomal vaccine, as did a combined treatment with pronase. These results suggest that ribosomal protein plays an important role in protection against this fungal infection.

AI-10449-03 - W. Johnson

This intriguing study is a continuation of exploration of the potentials of purified ribosomal fractions of Salmonella and streptococcal strains for immunization. A purified 50 S ribosome fraction from S. typhimurium showed good immunity in mice to challenge with a group B Salmonella; this immunity lasted for six months, suggesting that it is group-specific. The immunity appears to be mediated by a cellular response; it was found that lymphocytes, but not serum from immune donors, were capable of passively protecting non-immune recipients. Ribosomal preparations from S. pyogenes, with no detectable carbohydrate or M protein, also showed strong immunizing potential in mice against challenge ranging up to 1,000 LD_{50} of S. pyogenes. Enzymatic degradation of the ribosomal preparation with ribonuclease or trypsin showed a decreased survival rate when compared to controls immunized with untreated ribosomal preparations.

AI-01636-17 - G. P. Youmans

Purified RNA preparations from Mycobacteria were previously reported by this investigator to have a very strong protective effect in mice against lethal challenge by virulent strains. The immunogenicity of the mycobacterial RNA preparations for mice, however, was markedly inhibited by certain polybasic amines, e.g., protamine and neomycin. These compounds may have acted by masking the immunogenic sites on the mycobacterial RNA structure. Mice immunized with mycobacterial RNA showed no evidence, by the very sensitive MIF test, of tuberculin hypersensitivity. Dr. Youmans has also found that the mycobacterial RNA could function as a potent adjuvant to other agents for the induction of the primary immune response in mice.

Basic Microbiological Research

A bacterium that is a true parasite of another bacterial species has been the object of a continuing study by several research workers.

AI-08715-02 - D. Abram

Infection of the common intestinal bacterium E. coli by the parasitic bacterium Bdellovibrio serves as an interesting model for study of common processes related to membrane biology. Such processes as cellular penetration, cellular recognition, and membrane fusion can be readily studied by this unusual host-parasite system. Dr. Abram has found that the bonds between the host and parasite prior to penetration are electrostatic, and that parasite motility is not sufficient to initiate host infection. Shortly after the initial host-parasite contact the two different cells can no longer be disassociated, indicating formation of a very strong

bond. Electron microscopic study shows host cells which suffer a distinct, massive cell wall damage followed by protoplast dispersion. This is more pronounced when host cell penetration is prevented after the initial contact is made.

Glomerulonephritis

The etiologic and pathenogenetic events responsible for human glomerulonephritis are far from being well-defined. Further information is clearly needed regarding this important disease problem. It is hoped that eventually the information will make less imperative the need for renal transplantation.

AI-10334-04 - G. A. Andres

Among the more interesting observations by this investigator's group is the finding of a new type of cytotoxic renal injury produced by autoantibodies reacting with a tubular basement membrane of the kidney tubule. This lesion has been observed in patients with kidney transplants. Pathologic changes show that humans can develop autoantibody to the tubular antigens that are cytotoxic. Attempts to control this damage by pretreatment of the kidney transplant prior to surgery, with specific antibody fragments or with citrate perfusion, were unsuccessful. Treatment of liver failure by intermittent liver hemo-perfusion was performed on patients in deep liver coma, using various animal or human cadaver livers. Consciousness was restored in some patients, and two patients actually survived for more than two years post-treatment.

The specific and quantitative assay for antibiotics in vivo or in biological material has been a problem of considerable importance. Further therapy of a patient is dependent on the results of such assays to maximize the benefits obtainable from a given antibiotic.

AI-10741-03 - P. S. Lietman

This investigator has developed assays for some antibiotics, based on enzymatic (acetylation) modification of the drug catalyzed by an R-factor mediated enzyme. A previous assay was developed for gentamicin; now Dr. Lietman reports on a new assay for chloramphenicol. This assay can be carefully quantitated, with development of standard curves for as little as 1-50 mcg./ml. using only 0.01 ml. of biological sample. Specificity of the assay is excellent, and no interference is found in the presence of biological fluids -- urine, bile, CSF, or plasma. Both free and protein-bound chloramphenicol are measured. The new assay is exceedingly useful and is now used routinely in clinical laboratories.

C. BIOCHEMISTRY AND PHYSIOLOGY

The approximate level of support given Biochemistry and Physiology:

Type of Grant	Number	Amount
Research grants	341	$16,344,522[1]
Training grants	8	329,462
Fellowships	21	436,447

Summary

This branch continues its interest in basic and fundamental research problems which support the disease-related activities of the other branches. In pursuit of this, the branch receives and attempts to synchronize and orchestrate extremely divergent, but relevant, research grants from areas ranging from medicinal chemistry to molecular biology. Material from approximately 25 study sections is involved. Examples of research experiments follow.

General

AI-10806-01 - D. J. Tipper

Initial studies of an antifungal agent, thiolutin, are described. The sulfur-containing antibiotic, thiolutin, has been shown to be a potent, reversible inhibitor of the growth of the non-pathogenic yeast, Saccharomyces cerevisiae; viability was unaffected over the concentration range of 4 to 100 µg/ml. At concentrations as low as 2 µg/ml, the drug inhibited ribonucleic acid (RNA) and protein synthesis in whole cells and spheroplasts. At these low concentrations, protein synthesis continued for a short period of time after RNA synthesis was completely stopped. With higher drug concentrations (greater than 20 µg/ml) protein synthesis was inhibited; concentrations of thiolutin up to 100 µg/ml did not affect translocation or peptide bond formation in cell-free protein-synthesizing systems from yeast. The effect of thiolutin on the activity of partially purified dioxyribonucleic acid-dependent RNA polymerases was examined, and the drug was found to be a potent inhibitor of RNA synthesis in vitro. Inhibition was greatest when the polymerase was preincubated with thiolutin. Several mechanisms are discussed to explain the multiple effects of thiolutin on S. cerevisiae. Since the action of the drug is easily reversed, thiolutin may prove to be of use in studies of various stages of yeast growth which may have ultimate application to pathogenic agents in this group.

AI-02095-16 - C. P. Schaffner

The chemistry of the novel hexaene macrolide antifungal antibiotic, mediocidin, produced by Streptomyces mediocidicus has been under continued

(1) Includes 19 Career Awards.

investigation. The production of this antibiotic substance in the pilot plant represents the only world-wide source of this unique material.

Initial purification studies involving thin layer and column chromatography as well as countercurrent distribution liquid-liquid partition studies have revealed the presence of three major hexaene macrolide components. Comparative physical and chemical determinations have indicated that all three components are structurally closely related.

The approximated molecular weight of the mediocidins is 1447. They possess a single carboxyl and two nitrogen-containing groups. The fragment, dimethyl-2-methyl dodecanedioate, has been isolated after esterification of an oxidation product obtained from the perhydromediocidins.

It is suggested that the macrocyclic ring of the mediocidin components contains two polyenic chromophores - the obvious hexaene and a tetraene. This is consistent with evidence obtained with ultraviolet spectroscopy, quantitative hydrogenation, oxidation, and gas chromatography.

The mediocidin complex is a potent antimicrobial substance with many interesting biological properties. Several different biological studies with this antibiotic are currently in progress.

AI-02353-16 - W. Catlin

A chemically defined medium was developed that supports growth of various strains of Neisseria recently isolated from clinical material. Quantitative tests were made of seven strains of Neisseria gonorrhoeae cultivated in the defined fluid medium. When inoculated at concentrations of 2×10^5 - 6×10^6 cfu/ml, the gonococci multiplied with generation times $\geq$ 60 min, yielding about 5×10^8 cfu/ml within 10 hr and more than 10^9 cfu/ml by 24 hr. Defined agar media were used for investigation of the growth requirements of 146 strains of N. gonorrhoeae, Neisseria meningitidis, and Neisseria lactamica. Each species has a distinctive nutritional profile that serves to differentiate and identify it. On a series of defined media lacking different groups of compounds, the meningococci were relatively homogeneous in their growth responses and were more competent biosynthetically than strains of the other species. Gonococci exhibited a diversity of nutritional requirements; 74 strains were subdivided into 13 distinct clusters (auxotypes) on the basis of their patterns of growth responses to thiamine pyrophosphate, thiamine, proline, arginine, methionine, isoleucine, and hypoxanthine. Such results demonstrate the feasibility of developing a system for auxotyping of clinical isolates of N. gonorrhoeae.

Program Areas

(1) Mechanism of Resistance to Antimicrobial Agents.

During the past ten years, the importance to health problems of microbial resistance to therapeutic agents has become increasingly apparent. The relevance of this phenomenon to other interests of this Institute, such as hospital-acquired infections of staphylococcal origin and investigations of the gonococcus is obvious. At this point, there is substantial clinical and epidemiological evidence that development of this resistance, and especially plasmid (extrachromosomal) mediated drug resistance, represents a major problem in medical care. Since most of the projects dealing with the development of drug resistance are of a basic nature and have been concerned with defining the molecular and biochemical basis for resistance, the Special Emphasis program in this area has been transferred from the Bacterial and Mycotic Diseases Branch to the Biochemistry and Physiology Branch, and renamed.

The principal goal of this Special Emphasis program is to elucidate fundamental biological mechanisms involved in the development of drug resistance in microorganisms and to increase our basic understanding of this phenomenon. Specific goals of this Special Emphasis program involve investigation of the origin, development, evolution, expression and mechanism of drug resistance in a variety of specific microorganisms. Examples of microorganisms of particular interest are (but not limited to): species of gram-negative organisms and the enterobacteriaceae, staphylococci. Neisseria gonorrhoeae, pathogenic fungi, and the mycobacteria.

Projects of special interest to this program are included in one or more of the following major categories: 1) genetic characteristics, ecology, pathology, and epidemiological interrelationships of R factors and other drug resistance plasmids. 2) structural and molecular characteristics of drug resistance plasmids. 3) origin, development, and evolution of drug resistance in microorganisms. 4) replication and conjugal transfer of plasmids. 5) control of genetic expression of drug resistance. 6) mechanisms of antibiotic inactivation. 7) molecular basis of plasmid and bacteriophage control of toxin production.

Examples of research progress during the fiscal year follow.

AI-11297-02 - K. Takayama

This investigator has continued his studies on the mechanism of action of isoniazid (INH) on the tubercle bacillus. Current results indicate that the compound does not inhibit protein synthesis, but probably acts directly on an enzyme system consisting of mycolate synthetase (MS). It may be that the INH inhibition of MS activity is the primary mode of action of the drug. If this is the case, then the various characteristics of INH inhibition of MS activity on M. tuberculosis that have now been elucidated should become useful in devising more effective

means (with respect to dosage and treatment time) of treating the disease, tuberculosis, as well as rationalizing the drug's effectiveness (or lack of it) in specific cases.

When the mode of action of INH becomes known, other drugs with similar actions may be developed to cope with the disease, especially when dealing with the INH resistant atypical mycobacterial infections (assuming resistance is related only to lack of drug uptake).

These studies may eventually lead to the elucidation of the pathway for mycolic acid synthesis (a very important and predominant fatty acid component of mycobacteria).

AI-10646-02 - F. Sparling

A positive correlation was found between sensitivities of 147 random clinical isolates of Neisseria gonorrhoeae to all possible pairs of the drugs penicillin, tetracycline, erythromycin, chloramphenicol, acridine orange, and ethidium bromide. High-level resistance to streptomycin was also positively correlated with increased resistance to some of these drugs. This suggested that multiply-resistant strains are not exclusively the result of selection of independent mutants for resistance to each drug and that there may be a common mechanism for resistance to some or all of these drugs. This hypothesis was supported by the demonstration that low-level resistance to a large group of drugs (penicillin, tetracycline, erythromycin, chloramphenicol, rifampin, ethidium bromide, and acridine orange) could be lost as well as restored by a single mutational event in vitro.

AI-08619-07 - S. Cohen

Dr. Cohen has evolved a procedure for insertion of eukaryotic genes into E. coli which offers a general approach for the cloning of DNA molecules from various sources, provided that both molecular species have cohesive termini made by a restriction endonuclease, and that insertion of a DNA segment at the cleavage of the plasmid does not interfere with expression of genes essential for its replication and selection. The ability to clone specific fragments of DNA from the complex genome in prokaryotic cells provides a potentially valuable tool for the study of organization and function of eukaryotic genomes. It also provides a highly sensitive method for study of the molecular structure of bacterial plasmids.

In addition to the above, other investigations carried out concern the genetics, structure, and biochemical properties of an R factor system consisting of a conjugally proficient transfer plasmid and a naturally occurring non-conjugative plasmid. Despite their apparently widespread occurrence, relatively little had been published previously about the structural characteristics of the transfer and resistance determinant components of this type of R factor system. The plasmid transformation procedure developed was shown to be useful in specifically investigating the biological properties of discrete molecular species of plasmid DNA that are separable in vitro by sucrose gradient centrifugation, or by other fractionation procedures. In addition, transformation was shown

in these studies to be an effective means of inserting a non-self-transmissible plasmid into E. coli minicells, free from a transfer unit. The induction of tetracycline resistance carried by a small non-conjugative plasmid was studied in minicells and specific and reproducible changes in polypeptide synthesis patterns were demonstrated by polyacrylamide gel techniques in response to antibiotic exposure.

AI-11456-01 - R. Curtiss

During the past year, the mechanism by which the drugs rifampicin,chloramphenicol and nalidixic acid block the conjugal replication and transfer of plasmid DNA has been determined. In other studies he has isolated and begun a preliminary characterization of numerous mutants that specifically effect either donor or recipient ability and other mutants that effect regulation of vegetative plasmid replication. Hopefully these studies on transmission and function of plasmids that confer drug resistance will help in developing methods to curtail the spread of drug-resistance plasmids to pathogenic or potentially pathogenic microbes. Preliminary results in producing antibodies against plasmid-specified gene products afford a true opportunity to determine whether protective immunization against plasmid-specified gene products might not be a way to curtail problems due to infections with drug-resistant microorganisms. In addition, it is possible that these studies will lead to more rapid methods for the identification of microorganisms that contain drug-inactivating enzymes.

(2) Antiviral Substances

a) Interferon
b) Bacteriophage
c) Basic antiviral virology

One of these, interferon, is accorded special emphasis and currently this is not given special advantage in the competition for funds. It is recommended that grants including research on the structure of this substance and its mechanism of action at the molecular level should be given funding advantage at this point in time.

Only when the structure of interferon has been ascertained can meaningful attempts at synthesis be launched. Increased investigation of the molecular mechanism of action of interferon will lead, in turn, to a greater understanding of its mode of action against viruses. At this point very little is known about this and further research should be encouraged.

Research progress in this important program includes the following.

AI-09238-05 - W. B. Wood

Although assembly of subcellular structures of bacteriophage is an important aspect of growth and development, little is known about

the mechanisms or the genetic control of morphogenesis at this level in bacteriophage. Dr. Wood's studies are providing insight into these mechanisms. Bacteriophage T4 assemply appears to share some features of morphogenesis in higher organisms, in that neither can be explained entirely by simple self-assembly of structural components. The finding that phage non-structural proteins and host cell factors are required for assembly provides one of the first opportunities to investigate sophisticated mechanisms of morphogenesis in an experimentally favorable model system.

At this stage, the health-related implications of this work must be stated in general terms. Control of abnormal or deleterious growth, as in viral infection, will be aided by knowledge of the mechanisms by which growth normally occurs. More specifically, the studies on host-defective bacterial mutants promise to provide insight into aspects of the virus-host relationship connected with viral muturation that are now poorly understood.

AI-10947-03 - A. Kaplan

It has been discovered that some of the glycoproteins synthesized by herpes virus-infected cells contain covalently bound sulfate. Unlike the ester bound sulfate incorporated into cell-specific mucopolysaccharides, the sulfated glycoprotein synthesized by pseudorabies virus is completely resistant to the action of chondroitinase ABC. By means of proteolytic digestion studies, it was found that the sulfate is not incorporated into the same carbohydrate chain into which the aminosugar glucosamine (and consequently galatosame as well) is incorporated. It thus appears that pseudorabies virus synthesizes a novel mucopolysaccharide of a type not present in uninfected cells. This idea is supported by the observation that crude pronase preparations apparently contain a hydrolase which degrades the sulfate containing the segment of the virus-specific mucopolysaccharide to a small dialyzable fragment, a property which further differentiates the virus-specific mucopolysaccharide from those synthesized by the host cell. The nature of the moiety to which the sulfate is covalently bound is now under investigation.

The above findings suggest the possibility that the herpes virus genome can code for certain glycosyl transferases and perhaps a sulfo-transferase as well. It is likely that these transferases have properties which are quite different from host transferases, suggesting the possibility that highly specific, chemotherapeutically active inhibitors of the viral functions could be designed.

AI-10096-03 - C. Colby

The role of the cell nucleus in mediating the interferon-induced antiviral state of chick embryo fibroblast cytoplasms has been examined by means of autoradiographic detection of virus-specific nucleic acid synthesis. Treatment with homologous or heterologous interferon was carried out prior to or subsequent to enucleation. Enucleated cyto-

plasms were prepared by centrifugation in the presence of cytochalasin B (CB): physical enucleation using CB eliminates the necessity, with attendant uncertainties, for using actinomycin D as an effector of chemical enucleation during infection. Infection by the cytoplasmically replicating viruses, vesicular stomatitis virus and vaccinia virus, accompanied by autoradiographic detection of the acid-insoluble incorporation of tritiated nucleotides has enabled comparison of the activities of the two virion-associated RNA polymerases and of the vaccinia DNA polymerase in these cytoplasms. The data obtained indicate the necessity for cellular nuclear products in effecting interferon-induced antiviral responses.

AI-05629-11 - T. Merigan

Antigen-stimulated lymphocyte transformation, as measured by ^{3}H-thymidine incorporation, and interferon production were studied serially in humans after Herpes virus hominis (HSV) disease. Lymphocytes were isolated by filtration through a nylon fiber column and added to isologous macrophage cultures prepared 1 week previously. Macrophage-lymphocyte interaction was required for optimum interferon production. A selective increase in interferon production was associated with disease occurrence in some individuals. The increase in interferon production by lymphocytes was maximum between 2 and 6 weeks after disease. In contrast, lymphocyte transformation and serum antibody levels were similar throughout a 12-week period after disease and appeared to reflect prior immune status rather than acute infection. Some subjects with HSV disease failed to produce a detectable interferon response within the 2- to 6-week period after disease. In these individuals, lesion recurrence was more frequent when compared to subjects producing greater quantities of interferon after disease.

AI-11112-08 - R. Wagner

This investigator has conducted studies on the vesicular stomatitis virus which will lead to a better definition of host resistance to viral infection and may well pave the way to a rational approach to viral and tumor therapy and prophylaxis.

He has discovered that VS virus is synthesized in two cellular compartments: the cytoplasm and the plasma membrane. The nucleocapsid is made in the cytoplasm and the envelope, including the spikes, are made at the plasma membrane. These two separate components are then assempled to make progeny virions.

He has identified the VS virion transcriptase as the nucleocapsid L protein. He can dissociate and reassemble the nucleocapsid template with its enzyme, thus restoring the capacity to synthesize complementary RNA *in vitro*. These studies will enable him to dissect the events which control VS viral transcription.

He has also identified the lesion in group I VS virus RNA^- mutants as being in the transcriptase and not the template. Preliminary evidence suggests that the lesion in group IV RNA^- mutants is in the template. Reconstitution experiments should enable him to understand completely the genetic controls of VS virus RNA synthesis.

Isolation and partial purification of the protein kinase of VS virus has been accomplished. This enzyme is located in the envelope and phosphorylates the NS protein. This finding provides him with a powerful probe to study the function of the NS protein, which is probably involved in the fidelity of viral transcription and, possibly, in replication.

He is making progress in characterizing the glycoprotein and glycolipid of the VS virus envelope. These represent two important antigenic determinants that relate to immunity and to cellular antigenic markers.

Much of what he has set out to do has been accomplished. He is now in an excellent position to capitalize on his discoveries to provide a complete analysis of an important class of viruses.

Awards To Grantees

AI-11636-01 - S. Cohen

Beginning in 1945 the Passano Foundation has each year presented an award to a person or persons who made an outstanding contribution to the advancement of medical science and whose associated work was done in the United States. The Directors give prime consideration to work that has immediate clinical value, or that gives promise of practical application in the near future. This year the award will be made to one of our grantees, Dr. Seymour S. Cohen, who is one of the founders of biochemical virology. His pioneering studies of viral nucleic acid synthesis and especially his fundamental discovery of new enzymes in virus-infected cells provided a conceptual framework which profoundly influenced subsequent research on virus development and virus-cell interactions. As investigations of the pathogenesis of viral disease reach the molecular level, more attention is being focused on the critical role of viral enzymes and on the possibility of inhibiting their function as an approach to chemotherapy. Dr. Cohen's basic discoveries are thus likely to have increasing import in understanding and controlling viral disease.

AI-11839-01 - J. R. Kates

Joseph R. Kates (age 33), Professor of Microbiology and Chairman of the Department of Microbiology, Health Sciences Center, State University of New York, Stony Brook, is the 1974 recipient of the Eli Lilly and Company Award in Microbiology and Immunology.

Dr. Kates' contribution to science would be outstanding for a person of any age. In describing his contributions, two lines of experimentation in particular have had great impact in terms of influence upon research in the field of molecular biology of gene expression in animal viruses and eukaryotic cells. His most outstanding observation was the finding of a transcriptase (DNA-dependent RNA polymerase) as an integral part of the poxvirus virion. The ability of this enzyme to function utilizing a DNA template which was fully encapsidated within a viral protein coat was a "bubble-burster" to those who thought that DNA which was packaged in protein was "by definition" inactive. This finding also solved a dilemma concerning a requirement for gene expression in the uncoating of poxviruses. Since the initial finding of a transcriptase in vaccinia, many reports have appeared in the literature concerning the occurrence of various types of polymerases present in the virion of a variety of animal viruses. Probably most exciting among these reports were those by Howard Temin and David Baltimore, demonstrating the existence of a reverse transcriptase in the virions of the RNA tumor viruses. Dr. Kates' work has continued on poxvirus virion RNA synthesis. He has demonstrated that newly synthesized RNA chains are extruded from the varion by a mechanism which requires ATP and is not dependent on the continuation of the RNA synthetic reaction. Such a mechanism of transporting the RNA out of the particle may well be a general one among viruses and, of greater interest, may even account for transport of RNA away from the chromatin and out of the nucleus of eukaryotic cells.

Also emanating from some of these studies with the poxviruses was the realization that messenger RNA contains a rather large sequence of adenine residues. Previous to this work, it had been known for a number of years that poly A occurred in certain animal tissues, and that enzymes existed which could synthesize poly A from ATP. However, the earlier workers in this field did not believe that the poly A was part of any other RNA in the cell. Dr. Kates' work with vaccinia, and more recently with HeLa cells, has unambiguously demonstrated that poly A is attached covalently to many of the transcripts of cellular and viral genomes. In addition to poly A sequences, Dr. Kates has discovered the existence of guanine-rich sequences in the RNA of animal cells. These findings have led to a tremendous interest in the occurrence and role of these sequences in RNA.

A less well known but perhaps equally exciting portion of the research of Dr. Kates' group is the study of the structure and function of viral genomes which have been isolated from infected cells as DNA-protein complexes. He believes that further studies of these entities will increase the understanding of the active state of the viral genome in the host cell. In addition, these entities represent a simple model for chromatin.

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LBV
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D. PARASITOLOGY AND MEDICAL ENTOMOLOGY

The approximate level of support provided for parasitology and medical entomology in FY '74:

Type of Grant	Number	Amount
Research grants	169(1)	$6,702,000(1)(2)
Training grants	21	903,000
Fellowships	14	232,000(3)

The research grants were divided among the major groups of parasites as follows:

Parasite Group	Number	Amount
Protozoa	44	1,639,000
Helminths	68	2,748,000
Arthropods	57	2,315,000

The specific diseases supported most heavily by the research grants in this program are: schistosomiasis ($1,182,000), malaria ($528,000), filariasis ($343,000), leishmaniasis ($288,000), and toxoplasmosis ($253,000). Because of their importance as transmitters of diseases (such as malaria, filariasis and many viral diseases) and as serious human pests, more funds (approximately $1,413,000) are being expended this year on research on mosquitoes than on any other group of parasites.

Special Emphasis Areas:

Nearly one-half of the projects and of the amount expended continue to be assigned to the two Special Emphasis Areas: "Biological Regulation of Vectors" and "Immunity of Animal Parasites." Detailed analyses have been made of budget data and areas of emphases of the projects in these Special Emphasis Areas for use (along with other pertinent information being accumulated) in the forthcoming evaluation of these two programs. An *ad hoc* panel has been selected for the evaluation of the BRV program on June 17 and 18, 1974, and tentative plans provide for evaluation of the IAP program in October, 1974. Innovations are being made in these evaluations in an effort to improve on the evaluation of programs of this type.

(1) Includes projects awarded (6) and funds paid ($215,719, T.D.C. -- for new awards and restoration of budget cuts) from FY '73 Impounded Funds. Number of projects also includes 7 active (Type 5) projects for which FY '74 funds had been recommended but which have had current budget period extended at request of P.I. with result that no wards will be made from FY '74 funds.

(2) Amount is based on T.D.C. for awards made or being processed as of April 29, 1974, ($4,759,775) and estimated average of 40% T.D.C. for Indirect Costs.

(3) Includes 7 Career Awards.

(A) Biological Regulation of Vectors:

Recent findings of Reeves and his colleagues (AI-03028-15) illustrate two important features of our Extramural Program: (a) the essential unity of the program regardless of the assignment of projects for program administration and (b) the fact that under our present procedures not all work in Special Emphasis Areas is formally classified and identified with the appropriate area. This very productive project entitled "Ecology and Control of Arboviruses" is comprise of several sub-projects most of which focus primarily on various aspects of ecology of the arboviruses and thus is appropriately assigned to the Viral and Richettsial Diseases Branch. Some elements of the project however fall within the scope of vector-pathogen interactions and problems of vector control usually assigned to the Parasitology and Medical Entomology Branch. This emphasizes the importance of full staff cooperation in achieving success in the analysis, planning and evaluation of programs.

The problem of identifying and classifying all appropriate project and sub-project activities in such important categories as "Special Emphasis Areas" is specifically illustrated by developments in this project. These investigators have recently demonstrated the occurrence of two insect viruses in males, females, larvae and pupae of a colony of Culex tarsalis -- suggesting transovarian transmission. These viruses apparently cause degeneration of the salivary glands of the mosquito and thus could interfere with mosquitoes serving as vectors for arboviruses. Studies to confirm transovarian passage and the interference with virus transmission are underway. If interference with vector competence can be demonstrated, studies will be undertaken to elucidate the mechanism of interference and to determine how these microorganisms can be introduced effectively into field populations of vector mosquitoes. Such a system of biological control would have the advantage of leaving the mosquito's ecological niche occupied by a population of incompetent vectors rather than eliminating the mosquito and running the risk of having its niche filled by another disease vector.

Consideration should be given to identifying significant sub-project activity in the Special Emphasis Areas for maintaining a correct inventory of all appropriate work supported in these areas of high program relevance.

Recent developments in arbovirology (spread of dengue 2 virus to a number of the Pacific Islands and the potential for spread to many other countries, epidemics of "hemorrhagic dengue" -- the specific etiology of which is still uncertain -- in Southeast Asia, the reoccurrence of jungle yellow fever in Panama), together with the continued problem of vector resistance to pesticides, emphasize the urgency of research in this area.

The following examples are cited to illustrate the progress made in representative projects in the BRV program this year:

AI-11338-02 - D. J. Gubler

Studies have been made on the infection rates of eight geographic strains

of Aedes albopictus with dengue 2 virus. Evidence from the preliminary studies indicates that the Israel strain is highly resistant to infection (only 1 of 42 mosquitoes infected) and that the Viet Nam strain is quite susceptible (20 of 26 mosquitoes infected). The susceptibility of these two strains is being tested further to determine if lines with complete resistance and susceptibility can be selected. The objective of this research is to determine if dengue can be controlled by replacing a susceptible vector species with a refractory strain of the same species or by introducing the gene controlling resistance into the natural population. Studies are in progress, also on the physiological basis of resistance.

AI-10274-02 - A. Spielman

Spielman and a colleague have demonstrated that endod a butanol extract of the soapberry plant, Phylolacca dodeandra, which had been developed for the control of disease-bearing snails, may be even more effective for the control of disease-bearing insects. It is toxic at about one part per million to second and third instar Aedes, Culex and Anopheles mosquitoes. (Eggs and pupae are not affected and adults die only after ingestion of concentrated endod.) Thus, this extract is considerably more toxic than rotenone and about 100 times as active as commercial saponin preparation. The concentration of endod required to kill mosquitoes is less than that which kills snails and fish. In countries where the local manufacture of endod is commercially feasible, its use as an insecticide should be further evaluated. Field trials currently underway in Ethiopia on the use of endod (manufactured by the indigenous population) in the control of snail vectors of schistosomiasis and fascioliasis may result in concurrent control of medically important insects.

(B) Immunity to Animal Parasites:

Applying the recent striking developments in basic immunology, parasitologists and immunologists (often working together in teams) are making exciting progress in elucidating the mechanisms of immunity to parasitic infections, as the recent results of the following projects in this program illustrate:

AI-11289-02 - D. G. Colley

Dr. Colley has demonstrated that lymph node cells from mice infected with Schistosoma mansoni for 8 weeks, when exposed in vitro to an antigenic material from schistosomal eggs, elaborate a material which increases the migration of eosinophils 2 to 4 fold in an in vitro eosinophil migration assay. The lymphokine has been designated "Eosinophil Stimulation Promoter" (ESP). Work is proceeding on the characterization (physiochemically and biologically) of this lymphokine. This work contributes to the understanding of the function and mode of action of the eosinophil.

AI-08952-05 - R. Nussenzweig

In earlier work, Dr. Nussenzweig and her colleagues had demonstrated that the intravenous injection of small numbers of attenuated (X-irradiated) sporozoites induced a high degree of protective immunity and the formation of anti-sporozoite antibodies. In rodent malaria, they demonstrated considerable cross-protection and serologic cross-reactivity among sporozoites of different strains and species. In contrast, they found that antibodies produced against sporozoites of a species of simian and human malarias are highly species-specific, with cross reactions observed only among different strains of a given species. The sporozoite-induced immunity is highly stage-specific, with its effects mainly directed against the sporozoite stage, which undergoes antigenic changes during the process of development.

They have concluded that sporozoite-induced immunity is, at least in part, serum-mediated and that the spleen does not play an essential role in its development. Also, non-specific stimulation of the reticuloendothelial system does not play a very important role in protection against sporozoite challenge.

The studies are proceeding now on the role of cell-mediated immunity as part of the mechanism of protection, possible ways of enhancing the immune response, the nature of the interactions between sporozoites and normal/immune serum, and basic immunopathologic mechanisms in malaria. The fact that the immune response was much more variable and weaker in the primates tested makes it necessary to explore more fully the fundamental mechanisms of the response in the rodent system as a basis for further work which could lead to the development of a method for immunization of man against malaria.

The host-parasite relationships involved in the complete immune response to the various parasites are extremely complex, due in large part to the stages of development and/or the migration through various tissues and organs which characterize so many parasitic infections. Much work, therefore, remains to be done to characterize the frequently stage-specific antigens and to demonstrate the nature and quantitative response of various tissues to these before the primary long-range goals of this program can be achieved: more effective diagnostic procedures and practical methods for immunizing against major parasitic diseases of man.

While these long-range goals are being pursued, however, the size and nature of many parasites serve to make host-parasite models ideally suited for the study of many important basic problems in immunology and immunopathology, as the following examples illustrate:

(a) Rhesus monkeys infected with Trypanosoma rhodesiense have been shown to develop a glomerulonephritis which is related to selective deposits of reactant proteins of the alternate complement pathway (properdin system) and thus appear to provide a unique immunopathological model for the study of this important human disease.

(b) Several parasitic infections are now being used to elucidate the mechanism of granuloma formation, particularly with reference to the role of cell-mediated immunity to pathogenesis of the lesions. These include not only Schistosoma mansoni and capillaria hepatica, but also malaria parasites.

(c) Several helminth infections serve as excellent models for the study of the mechanism of immediate hypersensitivity reactions.

(d) The role of inflammatory reactions and the biochemistry and physiological causes of these in the expulsion of parasites from the host are being studied using Trichinella spiralis as the basic model.

(e) The changing host-parasite relationships which permit the emergence of opportunistic pathogenic infections from latent infections are being studied with such parasites as Toxoplasma gondi, Pneumocystis carinii, Trypanosoma cruzi and Strongyloides stercoralis.

These and many more intriguing questions are being addressed in the research currently supported by grants in this Special Emphasis Area.

Miscellaneous Parasitic Diseases:

Results of the following projects illustrate the diversity of parasitic diseases being studied in the general grants program in parasitic diseases.

AI-11277-01 - W. T. Hughes

In the first year of this project, Dr. Hughes has developed a tissue culture system using chick embryonic lung cells (CEL) or owl monkey kidney cells (OMK) which permits limited in vitro cultivation of P. carinii. He has succeeded in making three passages of the organism in cell culture, securing a three-fold increase in organisms. The cultivation of the organism is important in that it will provide a means by which a purified P. carinii antigen can be obtained to develop serologic tests for the diagnosis of P. carinii pneumonitis and in epidemiologic studies of this infection. Pneumocystis pneumonia is probably the most common cause of death of children with acute leukemia while in remission, and is common in other cancer patients not on therapy and in immunosuppressed persons generally. Little is known about prevalence of the infection in the general population or about its transmission.

AI-10588-03 - F. L. Andersen

Dr. Anderson has demonstrated that echinococcosis is highly prevalent in central Utah and should be considered a significant public health problem in that area. Of 164 dogs purged with arecoline hydrobromide, 37 (22.6%) were infected with Echinococcus tapeworms. Of 4,712 adult sheep examined at local abattoirs, 608 (12.9%) harbored hydatid cysts. Cooperative work with personnel from the State Department of Public Health and the National Center for Disease Control showed (by skin test

reactions) an incidence of 1.1 cases per 100 humans in the area of central Utah where the tests were conducted.

Laboratory studies have demonstrated that the larval stages of this parasite can survive considerably longer in intact liver tissue than in the free state, suggesting that exposed animal pits common in central Utah may be an important source of hydatid tapeworms to stray carnivores.

These studies, together with those of Schwabe in California (AI-06633) and Leiby in North Dakota (AI-07857) indicate that echinococcosis is much more widespread and a more serious public health problem than had been realized previously.

AI-11373-02 - G. M. Davis

Working in cooperation with the "Water-Borne Diseases Project" of the Smithsonian Institution (supported by U.S.-A.I.D. contract), Dr. Davis has made extensive collections of snails from various localities along with Mekong River in Thailand. These have been identified and curated to provide data for plotting the distribution patterns of hybrobidd taxa throughout the Mekong River to determine whether Lithoglyphopsis aperta exists in localities where dams are to be built. This snail, which has been demonstrated to serve as first intermediate host of the Schistosoma species infecting humans at specific locations on the Mekong River, has been studied in great detail and found to consist of at least three races. Other taxa suspected of close genetic relationships to L. aperta are being analyzed. These studies are essential for an understanding of the epidemiology of schistosomiasis in the Mekong River system (an endemic focus only recently discovered of a Schistosoma sp. whose relationship to other species of Schistosoma in the orient has not yet been fully clarified). It is very important that the biology and epidemiology of this species of Schistosoma be understood before the proposed dams are constructed along the river to avoid spread of the disease throughout the impoundments similar to the extension of schistosomiasis which has occurred in Africa with large river impoundments in recent years.

AI-07453-07 - G. R. DeFoliart

One of the most significant discoveries in the field of arbovirology during the past year was the demonstration by Defoliart and his colleagues at the University of Wisconsin that the insect-borne LaCrosse encephalitis virus is passed from one generation of mosquito to the next in the female's egg. This is the first demonstration of transovarian transmission of a mosquito-borne virus, a mechanism which probably explains the ability of the virus to survive freezing winters. The study also demonstrated that the virus can multiply in the mosquito larvae. Efforts to control the spread of this important human disease must take into account this new knowledge of its ecology and epidemiology.

Projects in the general parasitic disease program are indicative of the opportunities for use of parasite-host model systems for the study of some of the most challenging and enigmatic problems in biomedical science. Perhaps there is no better example than the area of mechanisms of intracellular parasitism discussed recently by Trager[1] on the basis of his work supported by this program (AI-00192, training grant; and AI-08989 and AI-10640, research grants). These models are valuable not only for studies on mechanisms of invasion, pathogenesis of disease and host response, but on such fundamental problems as the dynamics of biological membranes, one of the most active areas of research in the biomedical sciences.

Results from two projects in the parasitic diseases program illustrate the way in which findings may lead to developments of considerable importance in other quite unrelated areas of biomedical science. Dr. Linda Butler's work (AI-10636-02) on the photodynamic effect of light on dye-fed insects, undertaken to explore the possibility of control of insect pests with these chemicals, has shown that certain dyes approved for use in foods, drugs and cosmetics are photodynamically active. With the elucidation of the process of photodynamism and the use of test animals, evidence will be obtained on the potential for photodynamic injury to man and other mammals.

The use of the urease inhibitor acetohydroxamic acid (AHA) to study metabolic processes in gastropods suggested to Campbell (AI-05006-10) the potential application of this compound to the treatment of Proteus urinary infections of man and animals. In collaboration with Dr. Campbell, Dr. D. P. Griffith, urologist at Baylor College of Medicine, now has an active research program on the clinical use of AHA, (provided by Dr. Campbell, since it is not yet commercially available) which has been shown to be relatively non-toxic, to be rapidly excreted into the urine, and to prevent most of the pathology of Proteus infections and urinary stone formation in rats.

Finally, a follow-up note on an item in last year's annual report seems appropriate. At the recent Workshop on Long-Term Toxicity of Anti-schistosomal Drugs*, it was reported that preliminary evidence indicates that analogues of hycanthone developed on the basis of fundamental information relating the three-dimensional structure of the drug to anthelmintic activity and host toxicity developed largely by Bueding and his colleagues (AI-03515-15 and AI-08022-07) have indeed shown increased efficacy against the parasite and decreased mutagenic or carcirogenic activity in laboratory studies. It is also pertinent to note that

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(1) "Some Aspects of Intracellular Parasitism," Science, 183 (1974): 269-273.

*Sponsored by the U.S.-Japan Cooperative Medical Science Program Panel on Parasitic Diseases and Panel on Environmental Carcinogenesis, May 1-3, 1974.

Bueding's pioneering studies on the potentially toxic effects of this drug (hycanthone) have led to its use in the development of model systems for study of mutagenic, carcinogenic and teratogenic effects of chemicals, an emerging area of great importance in environmental health sciences.

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E. VIRAL DISEASES BRANCH

The approximate level of support given viral diseases:

Type of Grant	Number	Amount
Research grants	125	$6,511,741[1]
Training grants	26	1,258,771
Fellowships	14	158,191

Summary

The diseases caused by viruses continue to be a matter of great interest and of increasing importance in the health problems of man. The grant support programs on these virus investigations are designed to contribute to better ways of diagnosis, prevention and treatment of the variety of serious diseases these organisms cause. Each year good research leads develop that give hopes of discoveries that will lessen the severity of illness and reduce deaths caused by viruses.

Two new special emphasis programs, Clinical Virology and Influenza have been added to the two ongoing special emphasis programs of Viral Hepatitis and Chronic and Degenerative Diseases of Man. The Clinical Virology program was developed in response to an apparent need for a better understanding of the pathogenesis of human viral infections, knowledge of which is lagging behind contemporary discoveries in molecular virology. This program aims to merge the talents of young investigators well versed in the technologies and concepts of modern virology with those of clinical investigators, ideally in the setting of a hospital-based virus diagnostic laboratory.

The problems in influenza are more highly focused: there is an urgent need for a more thorough understanding of the ever-changing nature of the influenza viruses so that a truly effective vaccine can be developed to offset the catastrophic effects of a predicted pandemic in the early 1980's.

In the viral Hepatitis program improved methods of screening and prevention of infection are being investigated with promising results, and a better understanding of the long term effects and behaviour of the diseases has been obtained. Interesting advances are being made toward clarifying a number of the riddles of Chronic and Degenerative Diseases of Man.

Research Grants Highlights

Influenza

The only infectious disease still occurring in the United States in epidemic form in the winter is influenza. Research information obtained in

(1) Includes 7 Career Awards.

recent years is progressing quite well with leads towards the development of improved vaccines that will reduce or control future epidemics of this diseases.

AI-09304-06 - E. D. Kilbourne

In studies directly related to vaccine development the investigators have pursued their concept of infection-permissive neuraminidase specific immunization by development of an antigenically hybrid vaccine which contains the neuraminidase antigen of the more recent England-Hong Kong variant of flu. Sizable quantities of this experimental vaccine X-38a and a companion conventional vaccine X-37a are now available for testing. Further investigations are also being undertaken for development of better standardization procedures based on vaccine immunogenicity.

Studies have progressed on the isolation of polypeptides of influenza virus, contributory to a basic understanding of the virus, with potential for the production of vaccines containing the isolated purified protein antigens.

In addition this group has reinvestigated the recombination of the influenza B viruses not only with respect to the prospect of donation of high yield characteristic but also with respect to the feasibility of producing antigenic hybrids similar to those in use in the neuraminidase specific immunization studies with the influenza A viruses. Antigenic hybrid of influenza B viruses have been produced and the recombinational frequency is similar to that with the Influenza A viruses.

AI-05600-11 - P. Choppin

In these investigations a method has been devised for the isolation of the glycoproteins of paramyxoviruses with full recovery of biological activities using the non-ionic detergent Triton X-100. The finding that both neuraminidase and hemagglutinating activities reside on a single protein in the case of paramyxoviruses is in striking contrast to the situation with myxoviruses, in which the two activities are found on different virion proteins.

These studies on this grant have been in the forefront of the research on these viruses and their use as model membrane systems. This work has contributed significantly to our knowledge of virus structure and replication and membrane biogenesis. Such knowledge will hopefully lead to eventual control of diseases produced by these viruses. For example, the methods developed for isolation of the viral glycoproteins may be of direct application to vaccine production.

AI-08831-06 - R. G. Webster

These studies provide evidence that the hemagglutinin subunit on Hong Kong influenza virus, chief cause of the 1968-1969 epidemic, could have originated from an equine or avian source. The studies on recombination

in vivo provide evidence on the mechanism of how the new pandemic strains of influenza virus could arise. Immediate practical applications have been the techniques for identifying the surface antigens on influenza with monospecific antisera. Of particular importance for the future is the identification of influenza viruses in natural hosts that may serve as the source of influenza viruses for man.

Arboviruses

Potential of epidemics caused by the arthropod borne viruses continue to face those responsible for control of such outbreaks. Since environmental management prevents indiscriminate usage of pesticides and some of the carriers of these viruses are becoming resistant to certain pesticides, efforts are being undertaken to find other methods of control. Some encouraging leads are being followed in these investigations.

AI-00771-20 - T. H. Yuill
AI-11547-01 - W. H. Thompson

Investigators at the University of Wisconsin have demonstrated that the insect-borne LaCrosse (LAC) encephalitis virus is passed from one generation of mosquitoes to the next in the female's eggs. This mechanism - never before demonstrated in a mosquito-borne virus - probably explains the virus' ability to survive freezing winters. LAC virus, a member of the so-called California Encephalitis Group, has plagued parts of rural Wisconsin for about 20 years. The virus attacks young children primarily, causing severe illness with high temperature, headache, nausea, vomiting and convulsions. New knowledge of LAC virus gained in these studies may help efforts to control the agent.

AI-00984-14 - P. Galindo

Forty one species belonging to eleven genera of mosquitoes have been reported naturally infected with endemic viruses of the Venezuelan encephalitis (VE) complex that remains a threat to the Southwestern United States. Only two of these species of mosquitoes have been proved to be efficient natural vectors of VE and these are Culex (Melanoconium) aikenii and Culex (M) portesi. The preliminary observations on the bionomics and VE transmission ability of Culex aikenii seem to indicate that this species has the potential to become a formidable vector of the arboviruses. Its wide distribution and common occurrence near human communities warrants further study on the systematics, ecology and disease transmission potential of this mosquito.

AI-03028-14 - W. Reeves

In these investigations Culex tarsalis mosquitoes from a colony at Bakersfield, California were found to be infected with 2 noninclusion cytoplasmic viruses. Both viruses were polyhedral in form and with one almost twice the diameter of the other. The smaller agent has been passed serially in virus-free C. tarsalis to establish its viral nature.

Infection with the larger agent appears to reduce the survival period of male and female C. tarsalis by 2 to 3 days, and the agents are found in larvae and pupae as well as both sexes of adults which is believed to reflect transovarian infection. Salivary gland degeneration has been observed in a high proportion of infected females. Studies are in progress to determine if these viruses will interfere with infection by Western Equine Encephalitis and St. Louis Encephalitis viruses or if salivary gland degeneration will decrease the competence of C. tarsalis to transmit these arboviruses by bite and serve to reduce threat of epidemics caused by these mosquitoes.

Chronic and Degenerative Diseases

Reasonable explanations as to the means by which specific viruses persist in certain animals for prolonged times, some eventually causing slow degenerative disease, continue to require time-consuming research efforts in attempts to solve these problems. However, progress in these areas of research are beginning to shed some light on the role of viruses in relation to some of the chronic diseases of man.

AI-09023-04 - D. Walker

In studies to determine the prevalence of JC virus (virus isolated from tissue of patients with progressive multifocal leukoencephalopathy [PML]) it was found that infection is rather common among residents of Wisconsin, with most infections occurring during childhood. As yet, nothing is known about the character or the usual course of the primary infection, but in studies of a similar virus (papova virus) antibodies last for life in most infected animals and the infection is persistent.

If infection with JC virus is similarly persistent, PML could result when the latent infection is activated as a result of acquired immunologic deficiency or therapeutic immunosuppression. However, it could also result from viral invasion of the central nervous system during primary infection in an immunologically compromised adult. The results of the study do not permit a differentiation of the above alternatives but if PML results from an activation of latent infection, then a high proportion of the population, at least in Wisconsin, may be at risk if subjected to immunosuppression.

AI-06246-10 - J. G. Stevens

In investigations in rabbits which developed encephalitis after having been previously infected with herpes simplex virus (HSV), leads were obtained suggesting that the disease resulted from reactivation of a latent infection in the brain. Further studies now show that HSV can induce latent infections in both peripheral and central nervous systems of experimental animals. Thus, a critical, basic event required for support of the concept that the nervous system is the source of virus for recurrent cutaneous disease and some cases of encephalitis has been shown to occur. Application of these methods to human tissue has shown

that latent infections occur in the trigeminal ganglia. It now would be important to determine whether latent virus can also be detected in the central nervous system of man. Finally, the possible role of a latent viral infection in the genesis of chronic degenerative diseases of the central nervous system can now be seriously investigated in these experimental systems.

AI-09022-06 - A. Koestner

These studies are directed at utilizing the canine distemper complex as a model for pathogenetic studies of a virus-induced demyelinating encephalomyelitis where evidence has been accumulated relating multiple sclerosis to this disease. In light of this information, the successful experimental production, by these investigators, of demyelinating encephalitis, in gnotobiotic pups with a special canine distemper virus isolate has gained markedly in importance as a pathogenetic model of virus-induced demyelination.

Viral Hepatitis

In the past few years progress has been made in hepatitis research on the transmission to experimental animals, isolation and identification of both hepatitis A and hepatitis B viruses. However, much needs to be accomplished in clinical, epidemiological and laboratory investigations before a good understanding of this disease is accomplished.

AI-10894-03 - I. L. Schweitzer

The data obtained in these studies reinforce the concept that infection is transferred from mother to infant with high frequency when the woman has acute viral hepatitis B late in pregnancy or soon after delivery. When hepatitis occurs early in pregnancy or when the mother is an asymptomatic carrier, the attack rate for the infant is much lower. The infection may be transmitted from mother to infant in utero, at the time of delivery, or in the postpartum period. Long-term follow-up has revealed that, in most instances, once the baby becomes infected with subclinical hepatitis, it remains hepatitis B antigen positive for long periods of time. The most important aspect of these results, as it appears to the public's health, is that the mother-infant pairs add to the pool of hepatitis B virus within the community.

AI-10176-03 - P. F. Kohler

In these investigations it was found that infants born to mothers with either hepatitis B or the asymptomatic chronic carrier state frequently transmit the infection to their newborns at the time of birth. In further studies it was found that specific antibody treatment has apparently prevented this infection in three such infants at risk. Future studies are aimed at further clarification on this and other types of treatment of viral hepatitis cases.

Other Viruses

Investigations on clinical virology and such viral diseases as are caused by herpes, measles, mumps, poliomyelitis, rabies, respiratory syncytial virus, rhinoviruses (colds) and rubella continue to show progress.

AI-05577-09 - D. Horstmann

The impact of oral poliovirus vaccine on the circulation of enteroviruses was measured by means of virologic surveillance in two small New England cities. Sewage specimens, collected at weekly intervals over a seven-year period, revealed the presence of polioviruses regularly throughout the year with no seasonal variation. Other enteroviruses were recovered mainly in the summer and autumn, the same ones tending to appear each year. Characterization of poliovirus strains recovered from sewage and from children, using intratypic serodifferentiation and the temperature marker, showed the isolates to be largely vaccine derived. Apparently the continued use of oral poliovirus vaccine in the communities studied had had no apparent influence on the behaviour of other enteroviruses, but dissemination of the vaccine strains through contact infection probably contributes to rising level of herd immunity among susceptible children.

AI-09706-03 - H. Koprowski

During vaccine trails in persons without previous history of antirabies vaccination, administration of a single dose of vaccine provided an antibody response clearly superior to that expected from a single dose of duck embryo, or nervous tissue type vaccine. Administration of two, three or four doses of vaccine provided an immune response much higher than that expected following administration of fourteen or twenty-one doses of vaccines presently used. In persons vaccinated previously against rabies, single dose of the new vaccine provided a rapid and extremely strong antibody response. High virus neutralizing titers were observed three weeks after inoculation and persisted at high levels for several months.

AI-06864-08 - A. Schluederberg

In studies of measles immunity in children vaccinated before one year of age the results indicate that concern about vaccine failure in children vaccinated between nine and 12 months of age, while not misplaced, may have been overemphasized. Maximum protection against measles is to be expected by delaying vaccination until the first birthday, but there may be times when it is advisable to vaccinate at the younger age. However, when possible children vaccinated under such conditions and particularly those vaccinated before age nine months, should be revaccinated after one year of age.

AI-01023-17 - T. Weller

The little-understood natural history infection due to cytomegalovirus (CMV) in adults is a matter of increasing interest. That an occult infection may be present in the apparently normal adult blood donor is strongly implied by the occasional appearance of CMV mononucleosis in patients transfused with fresh blood. In a study of fifty donors the antibody patterns found suggest that the CMV-host relationship is more dynamic than heretofore appreciated. While not necessarily applicable to the normal adult population, the findings emphasized the necessity for critical interpretation of the clinical significance of titers of complement-fixing antibodies to CMV in donors.

III. RESEARCH GRANTS PROGRAM ANALYSIS

Fields	1974 Estimate Amount
Allergy and Immunology	$22,086,000
Asthma & Allergic Disease Centers	(1,347,000)
Lymphocyte Biology	(1,472,000)
Clinical Immunology and Immunopathology	(3,619,000)
Cell-Mediated Vs Humoral Antibody Responses	(1,302,000)
Bacterial and Mycotic Diseases	17,436,000
Streptococcal Disease and Sequelae	(1,165,000)
Mechanisms of Immunity to Antimicrobial Agents	(1,469,000)
Biology of Venereal Disease	(1,556,000)
Hospital-Associated Infections	(806,000)
Viral Diseases and Problem Areas	12,944,000
Antiviral Substances Including Phage and Interferon	(991,000)
Chronic and Degenerative Diseases of Man	(881,000)
Viral Hepatitis	(326,000)
Clinical Virology	(538,000)
Influenza	(701,000)
Parasitic Diseases and Problem Areas	6,977,000
Immunity to Parasites	(1,353,000)
Biological Regulation of Vectors	(1,667,000)
Subtotal	$59,443,000
International Centers for Medical Research	2,399,000
Total, Research Grants	$61,842,000

LVD

IDB

LM

GMB

LBV

COLLABORATIVE

RRB

TIB

COLLABORATIVE RESEARCH PROGRAMS

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COLLABORATIVE RESEARCH PROGRAMS

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

Annual Report for FY 1974

After a considerable number of changes in the organization and staffing of Collaborative Research during FY 1973, the past year has been relatively stable. There were however a number of changes in the Branches of Collaborative Research. In the Infectious Disease Branch Dr. James C. Hill joined the staff for the Bacterial Vaccine Program replacing Dr. Richard E. Horton who transferred to the Geographic Medicine Branch. Dr. Franklin Tyeryar replaced Dr. June Dunnick in the Hepatitis Program and assumed additional duties in the Influenza Program.

As to advisory committee activities the Infectious Disease Branch and the Transplantation and Immunology Branch continue to receive their outside scientific advice on program development and evaluation from their respective chartered advisory committees. The U.S.-Japan Program receives similar advice from the seven (7) panels which correspond to the U.S.-Japan Program components. Since the Research Resources Committee was abolished late in FY 1973 the Branch now receives its outside advice from consultants and ad hoc groups and by increased utilization of workshops. The International Centers for Medical Research (ICMR) Committee was also abolished late in FY 1973, and it is anticipated that the primary review of the International Centers for Medical Research renewal applications will be done by a special study section.

The NIAID Advisory Council reviewed the Institute's Collaborative Research Programs at the March 21, 1974 Council meeting. This type of review is consonant with the recommendations of the Program Mechanism (Cooper) Committee.

FY 1974 has witnessed a transition in budget formulation with the initiation of budgeting by scientific program activity as a substitute for budgeting by funding mechanism. The four NIAID program categories are Allergic and Immunologic Diseases, Bacterial and Fungal Diseases, Viral Diseases, and Parasitic Diseases. In Collaborative Research the entire Transplantation and Immunology Program is included under Allergic and Immunologic Diseases; the Research Resources Branch is spread over all major categories except for Parasitic Diseases; Geographic Medicine Branch covers all categories except Allergic and Immunologic Diseases. The program of the Infectious Disease Branch fit into the broad categorical programs of Viral Diseases and Bacterial and Fungal diseases.

During FY 1974 staff participated in a variety of training activities and seminars. During the summer of calendar year 1973 the staff participated in seminars at the Airlie House in Virginia during which time there was an opportunity for scientists from Extramural Programs NIH to meet and exchange ideas with counter-parts in Collaborative Programs. The Staff Training-Extramural Programs (STEP) modules sponsored by the Extramural Programs also

furnish an opportunity for participation on the part of Collaborative Research staff. A number of staff also participated in a Project Officer Training sponsored by the Office of Contracts and Grants. Administrative and secretarial staff had an opportunity to participate in training in procurement and personnel activities.

Special workshops continued to be utilized during FY 1974 as a means of focusing on new areas for potential development in Collaborative Programs. Participants in these workshops have included NIH Intramural and Extramural scientists as well as scientists from other Government agencies and from universities. Attendance has been limited as much as possible to individuals who were likely to contribute to the proceedings. Individual workshops are mentioned in the reports of the four branches. These workshops have proved to be a very useful mechanism for program development and evaluating the state of the art.

Research Resources Branch

The Research Resources Branch continued to change its directions from conventional reagents for research in virology to producing, testing and distributing more sophisticated reagents for isolated viral antigens, such as those antisera for the hemagglutinins and neuraminidases of influenza viruses; purified allergens including ragweed, ryegrass and ascarids; reagents representing the virion and associated antigens of hepatitis B and most recently immunologic reagents such as anti-IgE. Stepped up activities in production of allergen reagents has necessitated the establishment of an allergen certification laboratory. Through the Research Resources Branch's Interagency Agreement with the Atomic Energy Commission, the Molecular Anatomy Laboratory has been instrumental in the development and purification of hepatitis B reagents; in identifying internal and surface antigens which may be the basis for new respiratory syncytial virus reagents and, in association with investigators from Stanford University in demonstrating a DNA dependent DNA polymerase in the core of the Dane particle.

The Research Resources Branch works closely with the Center for Disease Control, the World Health Organization and various immunological societies in formulating and implementing reagent programs. Discussions are underway with the American Type Culture Collection in reference to possible transfer of selected virus seed stocks from the Research Resources Branch to the American Type Culture Collection.

Transplantation and Immunology Branch

The Transplantation and Immunology Branch (TIB) through techniques and its reagents program has contributed to research on a variety of diseases which may be associated with certain histocompatibility antigens. Candidate diseases include leprosy, psoriasis, ankylosing spondylitis, Hodgkins Disease, choriocarcinoma and multiple sclerosis. Studies on these diseases has been enhanced by techniques developed by the TIB contractors and by the use of research reagents which the program has furnished. At the same time the program has fulfilled its primary mission

of providing reagents and related methodology to improve the accuracy of tissue typing in organ transplants. The preloaded 72 well trays provided by the TIB are used to type for the majority of U.S. kidney transplants. Program supported research and development has now made it possible to freeze dry the many typing reagents in the Bank resulting in a more stable product which can be shipped with facility and economy.

The double blind study in a single large institution of the TIB developed antilymphocyte globulin (ALG) which will help establish the utility of this immunosuppressant. Although ALG has been used for a number of years this will be the first time that such a study has been carried out to study toxicity and efficacy.

The contract to study the immunopathology of kidney disease continues to yield important information on the etiology of Nephritis. The growing numbers of Nephrologists and transplantation surgeons attest to the utility of this research resource.

Although it is well established that tissue matching is useful in selecting the most appropriate donor for living related kidney transplants. there still is controversy concerning the utility of tissue matching when cadaver kidneys are used. In an effort to adequately study this complex problem the TIB has initiated a program to collect and analyze clinical and laboratory data on a large number of kidney transplants. Hopefully this study will deliniate the importance of the various antigens involved and thus facilitate transplantation of the large numbers of patients currently on paliative dialysis.

Despite best efforts to obtain compatible cadaver kidneys, it will probably always be necessary to perform transplantations across the histocomparability barier. Current drugs, while immunosuppressive are not selective: patients are protected against graft rejection and as a consequence they have an increased susceptibility of infectious disease and the metabolic problems of hypercortisonism.

The proposed new program entitled Immune Regulation is designed to find other immunologic means for abrogating the graft rejection. Hopefully this approach will prove more specific than the panimmunosuppression in current usage.

The rapidly growing field of immunology and the expansion of the serum program makes it necessary to develop programs for the prompt dissemination of information. The TIB programs of workshops and the annual publication of a catalog of reagents is designed to bring information on new developments, techniques and reagents to the attention of the Branch Scientist as rapidly as possible.

Infectious Disease Branch

In FY 1974 witnessed a marked increase in the activities of the Infectious Disease Branch (IDB) particularly in the implementation of a significantly enlarged influenza program and the initiation of a venereal disease program.

The latter is expected to increase in size and scope during FY 1975. Early consideration has been given to a program in hospital associated infections and it is anticipated that preliminary plans will be reviewed by the Infectious Disease Advisory Committee in October 1974 and by the National Institute for Allergy and Infectious Diseases (NIAID) Advisory Council in November 1974.

At the same time that there has been a marked increase in new program development and implementation, the pace of activities in hepatitis, bacterial and viral vaccines and antiviral substances continued unabated. There was also a continuing increase in the number and complexity of projects requiring review by the NIAID Clinical Research Committee (CRC).

The Influenza Program, in its present form, is the culmination of a set of recommendations to the Institute via the Infectious Disease Advisory Committee. Many of these recommendations emanated from the six Influenza Workshops over the past three years. The propensity of influenza virus to mutate has made prevention of epidemic disease a unique and costly problem. The Institute's Collaborative Influenza Program is designed to utilize new technology, particularly in the area of genetics to find a means of combating influenza. Since the disease does not occur in epidemic proportions every year, the program commitment is for an extended period of time. Probably the keystone of the entire contract activity, an Influenza Study Center, was initiated this year. Complimentary activities include genetic studies, development and evaluation studies on inactivated and attenuated live virus candidate vaccines, and eventually studies on antiviral substances in the prevention and treatment of disease.

Progress in hepatitis must be measured and reported in terms of combined Intramural and Collaborative efforts. Collaborative contributions are in the form of contracts for animal models, epidemiology, evaluation of hyperimmune gamma globulin, laboratory support, the Molecular Anatomy (MAN) Program and reference reagents and resources. Scientific and technical breakthroughs, generated in Intramural Programs, the MAN Laboratory or by NIH grantees can be readily accelerated towards clinical application by the system which has been built up over the past three to four years. There were two major breakthroughs in hepatitis research during FY 1974. First, was the finding of a DNA-dependent polymerase in the core of the Dane particle, giving added evidence that the Dane particle is the hepatitis B virion. Second was the demonstration using immune electronmicroscopy that a parvo-like virus is the cause of hepatitis A. These findings should lead to a new generation of tests that will help reduce the problems of hepatitis A and B.

The Antiviral Substances Program is continuing the clinical evaluation in man of exogenous interferon and adenine arabinoside in the treatment of various herpes virus infections. Results of these studies and a prior study to evaluate poly I·poly C in treatment of herpes virus keratoconjunctivitis are incomplete at this time. Program has maintained a network of interrelated projects on reagents, production methodology, assay systems and animal models for screening promising antiviral drugs. Significant progress was made in production methodology for exogenous interferon and the development of a sensitive reliable assay system for measuring interferon.

The projects in the Bacterial Vaccine Program are in various stages of progress. The pneumococcal pneumonia vaccine study is in the final stages of evaluation...field trials are underway and should be completed within two years. The bacterial meningitis vaccine program has progressed to the point of major field trials...a decision on this will be forthcoming in the fall of 1974. There will be a field trial of an inactivated mycoplasma vaccine in marine recruits in the summer and fall of 1974. At the same time, other candidate mycoplasma vaccines including temperature sensitive mutants are being studied in the Institute's Laboratory of Infectious Diseases. The streptococcal vaccine activities continue at the same limited pace since their initiation in FY 1968. Current efforts are focused on continued funding of the efficacy studies using available M protein and limited studies on obtaining more effective antigens through research on improved methods for purifying streptococcal M proteins. Projects in venereal disease and otitis media have just been initiated.

The Viral Vaccine Program includes studies on candidate vaccines for respiratory syncytial (RS) virus and parainfluenza virus infections. Previous efforts included rubella, rhinoviruses, and vaccinia. The rubella and adenovirus studies resulted in the development of successful vaccines; the rhinovirus effort has been abandoned and results of the study to determine an optimum dose, route and vaccine strain for smallpox immunization are still being evaluated.

Unexpected technical problems, including paradoxical responses to inactivated vaccines, have limited progress on development of successful vaccines for RS and parainfluenza infections. Since these viruses are causes of serious respiratory infections in children, efforts are continuing with emphasis on genetic manipulation of the viruses to produce stable, attenuated mutants for further testing as candidate vaccines.

Geographic Medicine Branch

The Geographic Medicine Branch includes two major spheres of activity; the U.S.-Japan Cooperative Medical Science Program and the International Centers for Medical Research (ICMR) Program.

Contracts in the U.S.-Japan Program support research resources activities, vaccine development and evaluation and projects to evaluate drugs in the treatment of leprosy and parasitic diseases. Malnutrition contracts are administered in conjunction with the National Institute of Arthritis, Metabolism, and Digestive Diseases (NIAMDD).

In the Cholera Program technical problems with the production of a satisfactory cholera toxoid have been resolved. The toxoid is now ready for testing in U.S. volunteers prior to shipping the material to Bangladesh for a full field trial beginning in July 1974. Dr. Willard F. Verwey has been appointed as Director of the Cholera Research Laboratory and will assume duties the first of June 1974. The program is also directing new efforts towards studying the magnitude of diarrheal disease due to E. coli and toward

possible future development of immunizing agents.

The Tuberculosis Program has provided valuable resources in the form of cultures and purified protein derivatives (PPD), and has administered a program to fractionate and define components of M tuberculosis. This is expected to lead to better immunizing agents or improved diagnostic methods for detecting infections.

As a result of NIAID collaborative support of a study of leprosy in a Pingelapese population, it appears that acedapsone (DADDS) has a beneficial effect in suppressing the disease and its spread.

Through the Parasitic Diseases Program, the Geographic Medicine Branch continues to provide researchers with animals infected with three human schistosomes and also makes available vectors and animal hosts experimentally infected with filarial parasites. Other efforts include those aimed at establishing cell lines from the snail host of Schistosoma mansoni. Success in these efforts has made possible cultivation of larval stages of S. mansoni itself. These resources and the complementary developmental studies should open new opportunities for studying and controlling parasitic diseases.

The Virus Diseases Program has essentially completed its studies on dengue virus with latest results indicating differences in the ability of various strains of Aedes albopictus to transmit dengue virus infection. Current emphasis in the program is focused on development of improved post-exposure rabies vaccines and on improved potency tests to measure their effectiveness in vitro.

The Malnutrition Program operated conjointly with the NIAMDD sponsored a Conference entitled "The Latent Effects of Malnutrition and Infection During Pregnancy as Determinant of Growth and Development of the Child." Among such discussions at the Conference were the significance of asymptomatic bacterial infection during pregnancy. A program-sponsored study has suggested that bladder stones may be the outcome of malnutrition in Thailand. It was also demonstrated in Thailand that pneumonia, otitis media, gastro infections and susceptibility to hepatitis B are also related to malnutrition. The program produced a comprehensive reference entitled "Food Composition Table for use in East Asia."

The newest Panel in the U.S.-Japan Program deals with Environmental Mutagenesis and Carcinogenesis. They are focusing on the potential danger of AF-2, a nitrofuron compound commonly used in food products in Japan. Included in their work is an assay system using drosophila as an indicator of mutagenic and carcinogenic activity.

The International Centers for Medical Research (ICMR) Program which supports four centers through the grant mechanism is currently being reviewed as part of the five year cycle. During the past year the Johns Hopkins University Center has moved its off-shore laboratory from Calcutta to Dacca, Bangladesh

and the University of Maryland which has its primary site in Lahore, West Pakistan is now phasing out a field unit established in Salvador, Brazil which had been established in FY 1971. The University of California and Tulane University remain in Kuala Lumpur, Malaysia and in Cali, Colombia, respectively.

LVD
TDB
LM
GMB
LBV
L&CR
RRB
TIB

RESEARCH RESOURCES BRANCH
PROGRAM STATUS REPORT

The Research Resources Branch (RRB) conducts a program for the support of research by the production, testing and distribution of a wide range of seed viruses, mycoplasma, allergens and corresponding antisera to research scientists. The branch identifies reagents for which a critical need exists, has these produced through the contract mechanism, distributes them as reference reagents and disseminates information and technical advice on reagent characterization and use.

Enteroviruses

Seven additional Lim Benyesh-Melnick intersecting typing pools (J-P) were processed and packaged during the past year. These new reagents are presently being tested by the certification laboratory and will subsequently be added to an array of eight other pools (A-H) presently being distributed. These pools receive wide distribution and continue to be an important adjunct to the rapid identification of members of this important group of viruses.

There are sixty-eight enteroviruses now available for distribution as sets of seed virus and antiserum preparations. The past production activities have resulted in this complete set of recognized serotypes and in the future production of new serotypes will be undertaken only when appropriate. A new candidate serotype (enterovirus 69) Toluca-1 is in production under an interagency agreement with the Center for Disease Control (CDC). Following assay by the certification laboratory it will receive wide distribution for verification of its uniqueness. The certification laboratory continues to be located at the California State Department of Public Health and in addition to certification activities is also involved in the identification of new types and testing related to the long term storage behavior of selected reagents.

Arboviruses

The major objective of this program element is rapidly nearing completion, i.e., the production of seed virus and monospecific antibody preparations for those arboviruses of demonstrated or potential importance to disease on the North American continent. The certification laboratory for this program is located at the Yale Arbovirus Research Unit and serves as an independent source of data for verifying production specifications and adding other pertinent data to fully characterize and catalog the bulk and packaged materials supplied by other contractors.

During the past year two production contractors completed reagents for St. Louis Encephalitis, Russian Spring Summer Encephalitis, Japanese B Encephalitis, Powassan, Semliki Forest, Piry, Mucambo, Venezuelan Equine Encephalomyelitis (Fla.), Dengue 2, and Dengue 3 viruses. During FY75 one production contractor will produce reagents for Lymphocytic choriomeningitis, Bocas, Dengue 1, and Dengue 4; it is anticipated that these reagents will complete the array of single virus sets for the arbovirus collection.

This program also supports the Catalog of Arthropod-Borne Viruses of the World through an interagency agreement with the CDC at its Ft. Collins, Colorado Laboratory for Ecological Investigations. This is a very useful mechanism whereby research laboratories exchange information on new as well as established arboviruses. This activity currently has more than 320 viruses catalogued and there are approximately 150 participating laboratories throughout the world. RRB will support the publication of a formal published catalog of these viruses during FY75. Support will be provided through the interagency agreement with CDC and the principal investigator, Dr. Trygve Berge, will serve as editor-in-chief.

During FY74 the production of three mosquito blood meal antisera were completed. These were for the identification of three taxonomic groups of birds (heron, pigeon, and sparrow/starling) and the reagents are being assayed by a recently established certification laboratory at the Florida Department of Health's Entomology Laboratories in Vero Beach. During FY75 three additional order-specific sera (reptilian, amphibian, and chicken) will be made and will complete the array of such sera.

The Mammalian Identification Service at the Smithsonian Institution will no longer receive support from RRB at the end of the current period. In the past this laboratory has provided a central source for taxonomic identifications for investigators conducting field and laboratory studies. Primarily this service was used by laboratories in Central and South America and resulted in several sicentific papers and keys which will now serve as a permanent source of classification information.

Rhinoviruses

The contractor at the Children's Hospital Research Center of Ohio has provided data that there is an expanding array of serotypes of viruses within this group going beyond the three phases presently described. Based on this information, production of sets of seed viruses and antisera will be terminated with the expiration of the one remaining production contract at the University of Wisconsin. This will result in reagents for Phases I and II for a total of 89 viruses.

The certification laboratory is at the University of Vermont and this laboratory provides the data necessary to characterize all reagents in both bulk and packaged forms. The certification laboratory is also involved in preparation of data for the rhinoviruses in catalog format. It is anticipated that the certification contractor will complete all work during FY75.

Myxovirus

For the second consecutive year no seed viruses or antisera were produced for any of the myxoviruses. However, a contract for the production of monospecific antisera for all the known neuraminidases and hemagglutinins of the influenza viruses of man and animals has entered into its second year. Work on these sera is proceeding on schedule and at the completion of production investigators will have available monospecific antisera for eight neuraminidases and fifteen hemagglutinins for use as reference reagents in support of their studies in research and epidemiology.

A previously acquired small supply of a synthetic neuraminidase substrate 2-3'(methoxyphenyl)-N-acetyl-α-neuraminic acid (MPN) was rapidly committed. This substrate made possible a direct colorimetric assay of this enzyme of great importance in the study of the influenza virus. No commercial source of the material is available, therefore, the award of a second contract is imminent to provide 20 g of MPN to fulfill an established and continuing need for this reagent.

Adenovirus and Adenovirus Associated Viruses (AAV)

During FY74 no production activity was in effect for these viruses. However, RRB did sponsor a Workshop on Parvoviruses that was chaired by Dr. M. David Hoggan of LVD, NIAID. This workshop brought together 24 scientists for a two-day meeting to allow them to assess progress and devise new approaches to research on this diverse group of organisms. The AAV, the virus of non-bacterial gastroenteritis and hepatitis A will have greatest interest to RRB.

During early FY75 this program element will acquire antisera to three serotypes of AAV, through cooperation with Dr. Hoggan, and these will complete the array of reagents for these viruses.

Herpesviruses

During FY74 the Research Resources Branch sponsored a workshop on these viruses to identify the need for and feasibility of producing reagents. This meeting was attended by 65 investigators who heard 24 formal presentations about these important viruses. Primary interest and discussion centered around herpes simplex types 1 and 2, the cytomegalovirus, and the Epstein-Barr virus. The final report of this meeting will serve as a guideline for Branch Consultants as they develop an expanded program for these viruses early in FY75.

Hepatitis

Research Resources Branch in cooperation with the Infectious Diseases Branch sponsored a Workshop on Hepatitis B during FY74. This afforded the opportunity for 53 researchers to meet and discuss various aspects of their work including: antigenic subtypes, the characterization of the Dane particle, the biological characterization of the infectious unit and immunohistochemical assays.

Two additional hepatitis B reagents were acquired for distribution during FY74. The AEC-MAN laboratory produced a rabbit anti-HB_sAg/adr antiserum and the Bureau of Biologics, FDA provided a rhesus monkey antiserum reactive against the core components of the Dane particle. These augment the subtype reagents previously acquired and expand the array to cover the following antigens: HB_sAg/adw, HB_sAg/ayw, HB_sAg/adr, and HB_cAg.

Developments with hepatitis A are being closely monitored to identify the need for and opportunity to produce reference reagents for the newly-demonstrated virus.

Allergens

During the last month of FY74 it is anticipated that work on the production of antigens E, K, and Ra3 from 20 kilograms of short ragweed pollen will be completed, the antigens packaged, and distribution begun early in FY75. These materials are being prepared and packaged for distribution as an Investigational New Drug. These antigens should serve an established need for such materials for a prolonged period of time.

A contract for the extraction and packaging of Group Antigens I, II, III from 10 kilograms of ryegrass pollen has been in effect for nearly one year. Technical problems have been encountered when scaling up the procedures from laboratory to production operations and presently the work is behind the initial time estimates. It is anticipated, however, that the products will be acquired during FY75.

A contract with Alpha Gamma Laboratories for the production of 2 liters of a polyvalent antiserum against the antigens of ragweed pollen is nearing completion. When packaged for distribution this antiserum will provide a long-term reference material for serologic assay of the predominant antigens in this pollen of importance to allergic disease.

The allergen Asc-1 from Ascaris suum has been demonstrated to produce high levels of IgE in laboratory animals and, therefore, is of importance to allergy research. Accordingly, Research Resources Branch awarded a contract to the University of Western Ontario to produce 500 mg of this material. Work is proceeding on schedule and delivery is expected midway through FY75.

Award of two additional contracts associated with the RRB allergen program is anticipated during the last month of FY74. One contract is to produce an antiserum against human IgE. This reference reagent will be particularly useful to laboratories conducting the radioallergosorbent (RAST) test as a research and diagnostic assay for allergic diseases. The second contract for award will be to establish a central allergen certification laboratory to serve as a long-term facility for the expanding allergen program of the Branch.

AEC-NIAID Molecular Anatomy Program (MAN)

The Rockville Laboratory of the MAN Program is currently in its fifth year of operation as a cooperative project of the National Institute of Allergy and Infectious Diseases (NIAID) and the U.S. Atomic Energy Commission (AEC). The major objective of this program remains the development of methods for isolating viruses and viral antigens and the application of these techniques to research on associated diseases. The training of NIAID intramural scientists and contractors in the research application of specialized instrumentation developed by the MAN program, particularly the zonal centrifuge, is an important aspect of the laboratory's mission.

Current research programs of the Rockville laboratory include: (1) immunochemical characterization of the recognized subtypes of hepatitis B antigen (HB_sAg), (2) preparation of subtype-specific hepatitis B reagents for the

RRB, (3) the use of HB_sAg-specific DNA polymerase as a marker of hepatitis B virus infectivity and characterization of the DNA product of the polymerase enzyme, (4) development of a zonal centrifuge purified and inactivated trivalent parainfluenza vaccine, (5) isolation and characterization of structural antigenic components of Respiratory Syncytial Virus (RSV) and its temperature sensitive mutants, and (6) biophysical studies with the virions associated with hepatitis A and non-bacterial gastroenteritis.

The most significant single finding by this program during FY74 related to the studies of DNA polymerase activity. DNA polymerase activity was detected in each of eight preparations of concentrated human HBAg rich in Dane particles prepared by high-speed centrifugation of antigen-positive human plasma and in none of seven control preparations prepared in the same way from HBAg-negative plasma. The incorporation of ^{3}H-thymidine-methyl-5'-triphosphate into DNA was dependent on four deoxyribonucleoside triphosphates and $MgCl_2$. Treatment of the concentrated HBAg preparations with the nonionic detergent Nonidet P-40 (NP40) more than doubled the enzyme activity. Fractionation of the concentrated HBAg preparation in sucrose density gradients after treatment with NP40 revealed that the enzyme activity appeared within the density range of Dane core antigen but at a slightly higher density than the average for core antigen. The only particles observed by electron microscopy in this region of the gradient were typical 28-nm cores, suggesting that the DNA polymerase activity was associated with a subpopulation of cores. No DNA polymerase activity was found in purified 20-nm HBAg particles. The DNA product of the reaction remained associated with the 110S core and was not susceptible to DNase digestion when associated with the core. Inhibition of the reaction by actinomycin D and daunomycin suggested that the reaction was dependent on a DNA template associated with the core. These findings are consistent with the hypothesis that the 42-nm Dane particles are hepatitis B virions.

Research and development at Oak Ridge in support of the Rockville laboratory includes continuing efforts to increase the efficiency and biocontainment of zonal centrifuge separations, to optimize automated recycling immunoadsorption chromatography systems for mass production of specific antibodies or purification of trace antigens, and to investigate new ultra-sensitive modes of antigen detection.

Processing and Distribution of Reagents.

In addition to the various program elements detailed above, RRB also distributes reagents for mycoplasmas, coronaviruses, reoviruses, and interferons. In past years RRB also had a collection of simian viruses but during late FY73 donated this collection to the WHO International Reference Center for these viruses at the Southwest Foundation for Research and Education. The RRB reagent collection now includes 738 individual reagents. The repository and distribution contract remains at Flow Laboratories, Rockville, Md. and a tabular record of distribution by that facility since FY68 follows:

FISCAL YEAR	TOTAL TRANSACTIONS	TOTAL AMPS & VIALS DISTRIBUTED
1968	391	31,776
1969	402	15,805
1970	474	26,242
1971	638	12,902
1972	572	21,801
1973	575	19,181
1974 (9 months)	381	8,345

During the first three quarters of FY74 the distribution of reagents by groups was as shown in Table I.

The processing contractor is the American Type Culture Collection and work is now proceeding at the optimal rate. This contract is a key part in the RRB program since the expertise of the processing laboratory is relied on to prevent degradation of reagent quality during processing. Results of this contract are satisfactory and the contractor has solved all problems cited in the FY73 Annual Report.

TABLE I
RESEARCH RESOURCES BRANCH
DISTRIBUTION OF SPECIFIC VIRUS GROUPS
July 1, 1973 - March 31, 1974 - (3/4 year)

# of Items Avail. (% of Inventory)	Virus Group	SHIPMENTS # of	SHIPMENTS % of	AMPOULES # of	AMPOULES % of # Distributed
62 (8.4)	Adenovirus	12	3.1	191	3.5
154 (20.9)	Arbovirus	16	4.2	104	1.9
229 (31.0)	Enterovirus	66	17.3	797	14.7
6 (.8)	Hepatitis	64	16.8	306	5.7
15 (2.0)	Herpesvirus	9	2.4	23	.4
5 (.7)	Interferon	60	15.7	101	1.8
55 (7.5)	Mycoplasma	25	6.6	362*	6.6
63 (8.5)	Myxovirus Paramyxovirus and related	39	10.2	246	4.6
4 (.6)	Ragweed	30	7.9	144	2.6
6 (.8)	Reovirus	4	1.1	21	.4
139 (18.8)	Rhinovirus	11	2.9	201	3.7
	Combinations	45	11.8	2,909	54.1

Figures based on 381 shipments
5405 ampoules shipped (excludes testing, packaging, and WHO)

*Does not include one WHO shipment totaling 2940 ampoules.

INDIVIDUAL CONTRACTS AND AGREEMENTS TABULATION
RESEARCH RESOURCES BRANCH

VIRAL REAGENTS PROGRAM

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
NO1-AI 90089	California State Dept. of Public Health	To serve as a certification laboratory for all enterovirus reagents. During current year contractor is certifying LBM pools J-P and Toluca-1 reagents	12/11/71	33,904	Renewal
1-YO1-AI 40004-00	Center for Disease Control	Production of reagents for the enterovirus Toluca-1. Seed virus production is nearly complete and antiserum production will follow.	2/1/74	9,600	Renewal
NO1-AI 60460	Yale University	To certify all bulk and final packaged arbovirus reagents. During current fiscal year contractor has tested 54 bulk and final packaged reagents.	1/19/66	70,137	Renewal
NO1-AI 80679	California State Dept. of Public Health	To produce seed virus, immune ascitic fluids and control ascitic fluids for selected arboviruses. Since last annual report contractor has completed work on 8 reagent sets.	4/22/68	66,060	Expiration

VIRAL REAGENTS PROGRAM (CONT'D)

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
NO1-AI 32001	University of California	To produce antisera against the blood meals of arthropods important as vectors of arboviruses. During FY74 contractor was helpful in various aspects of the production of six antisera	11/01/72	33,380	Expiration
NO1-AI 42421	Florida Dept. of Health	To certify arthropod blood meal reagents.	5/1/74	6,613	Renewal
NO1-AI 70059	Smithsonian Institution	To identify mammalian specimens submitted by laboratories involved in ecological studies related to viral diseases with special reference to arboviral diseases	12/01/71	19,592	Expiration
2-YO1-AI 2007-03	Center for Disease Control	To maintain a registry of data from investigators on arthropod-borne viruses. The catalog currently contains data for 331 viruses.	10/01/71	29,940	Renewal
NO1-AI 22517	University of West Indies	To investigate the feasibility of preparing rat ascitic fluids for Dengue serotypes. Results demonstrated that the rat did not offer significant advantages as a producer of fluids when both antibody activities and fluid volumes were concerned.	06/27/72	prior year support	Expired

VIRAL REAGENTS PROGRAM (CONT'D)

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
NO1-AI 71188	University of Vermont	To test, identify and certify rhinovirus reference reagents. Contractor is completing certification of bulk and final packaged seed viruses and antisera for 89 rhinovirus serotypes.	06/30/67	45,779	Expiration
NO1-AI 12111	University of Wisconsin	To produce five seed viruses and one antiserum for use as rhinovirus reference reagents.	04/27/71	prior year support	Expired
NO1-AI 92062	Children's Hospital Research Foundation (Ohio)	To determine which untyped rhinoviruses in contractors collection are not members of previously described serotypes. Results indicate that there are at least 125 serological types of rhinoviruses.	05/01/69	18,953	Expired
NO1-AI 32516	St. Jude Children's Research Hospital	To prepare monospecific antisera to the isolated hemagglutinins and neuraminidases of influenza viruses. Approximately half of the antisera were produced during this fiscal year.	03/22/73	44,286	Expiration

ALLERGEN REAGENT PROGRAM

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
NO1-AI 42500	University of Western Ontario	To produce 500 mg of allergen Asc-1 from Ascaris suum.	10/15/73	10,600	Expiration
NO1-AI 32534	Stanford Research Institute	To produce and package the Group I, II, and III allergens from ryegrass pollen.	06/28/73	prior year support	Expired
NO1-AI 32533	Alpha Gamma Laboratories	To produce 2 liters of polyvalent rabbit antiserum against short-ragweed pollen antigens	06/29/73	prior year support	Expired
NO1-AI 32507	Worthington Biochemical Corp.	Produce and package antigens E, K, and Ra-3 from 20 kilograms of short-ragweed pollen.	10/2/72	prior year support	Expired

AEC-NIAID MOLECULAR ANATOMY PROGRAM

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
2-Y01-AI 00002-06	Atomic Energy Commission	Preparation of subtype reagents for HBAg and purification of Dane particles from serum. Collaboration in animal model studies and preparation of candidate hepatitis vaccines from selected sources of HBAg and characterization of HB antigens found in diseased tissues and cell cultures. Biochemical studies with RS virus and development of radio-immunoassay for antibody to RS virus. Preparation of purified inactivated trivalent parainfluenza experimental vaccines and stability studies on pilot preparations. Biophysical characterization of agents involved in acute infectious non-bacterial gastroenteritis and hepatitis A. The most significant finding was that a DNA polymerase is associated with Dane particles and a subpopulation of the cores. That finding is consistant with the hypothesis that the 42-nm Dane particles are hepatitis B virus.	4/24/64	460,432	Renew

PROCESSING & DISTRIBUTION PROGRAM

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
NO1-AI 42515	American Type Culture Collection	To process bulk reagents and package them in final, standard form.	2/28/74	64,410	Renew
NO1-AI 22515	Flow Laboratories	To serve as a repository and distribution facility for all reference reagents.	5/1/72	115,655	Renew

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Part B - Staff

None

LVD
LM
LBV
LMI
TDB
GMB
L&CR
LCI
TIB

TRANSPLANTATION AND IMMUNOLOGY BRANCH
PROGRAM STATUS REPORT

During fiscal year 1974, approximately 2.4 million dollars were spent on contracts and agreements in support of Transplantation and Immunology Programs. The goals of the contract program are to develop and coordinate methods, procedures, and resources to enhance research in transplantation and immunology and to develop and utilize new procedures for the evaluation and monitoring of such programs. These efforts can be considered to fall into six general program categories.

I. Reagents and Techniques Program

Since its inception in 1965, much progress has been made by the Collaborative Transplantation and Immunology Program in developing the methodology for histocompatibility typing and in making quality reagents available. The nine contracts in this field are to: compare various tissue typing techniques; develop new typing techniques that are simpler or more accurate than those currently employed; produce a continuing supply of high-quality histocompatibility typing sera; conduct research directed at discovering new antigen specificities; and, to provide other reagents used in immunology research.

Refinements of typing techniques have been studied by four contractors. A comparison of cross-matching techniques is underway to determine the most reliable procedure. A controlled study of the efficacy of lymphocytotoxicity and fluorochromasia techniques has been completed. The platelet complement fixation test has been shown to be a simple, easily readable typing technique, which can be automated. The importance of mixed lymphocyte culture reactions in the matching of donors and recipients is being evaluated, as are several other procedures designed to detect immunologic discrepancies between individuals. Neutrophil specific antigens have been discovered; antisera have been produced, and made available for study in bone marrow and other transplants. Improved techniques and reagents have resulted in the discovery of new histocompatibility antigens. Several of the typing contractors have made contributions in this area.

The NIAID typing serum bank currently contains over 1,300 sera with a total volume of 268 liters. Additions to the bank in FY'74 included 78 sera obtained by contracts from four laboratories, 72 donated by users of the bank and 17 purchased outright by the Branch. Seven typing laboratories performed quality control analyses of the sera prior to their acquisition for the bank, thereby increasing the quality of the sera in the bank. Usage of the bank remained heavy with 1,275 shipments to 314 investigators during FY'74.

Through the use of a standardized tray and uniform lymphocytotoxicity typing techniques, a continuing effort is made to maintain the quality of reagents and the reproducibility of laboratory results. Over 19,000 trays

were delivered to over 116 U.S. laboratories for clinical organ typing during FY'74. Significant improvements in the clinical typing trays have been made by increasing the number of sera from 60 to 72, now defining 32 specificities.

Work continued during FY'74 to produce and distribute trays filled with lymphocytes from well-characterized individuals. These will provide a standard profile of histocompatibility antigens for use in quality control of sera, and for the assessment of patient's sensitization.

Other immunological reagents have been produced by TIB contractors; human complement components and inhibitors have been made available to qualified investigators, as have soluble mouse histocompatibility antigens (described in Section V). In addition, a new contract is under consideration to produce rabbit monospecific human HL-A xenoantisera.

II. Evaluation of Tissue Matching in Renal and Bone Marrow Transplantation

Although it has been demonstrated that histocompatibility matching improves the chances for success in living related kidney transplants, no conclusive data have been obtained concerning the importance of matching in transplants involving cadaver kidneys. Although preliminary reports are encouraging, they deal with small numbers of transplants, with little clinical data, and with incomplete typing information. A very large investment is being made by the NIAID and a number of other government agencies in providing reagents and services to type for the estimated 2,000 cadaver kidney transplants which are performed each year in the U.S. when there is a paucity of information concerning the importance of such a procedure. With this in mind, the Advisory Transplantation and Immunology Committee of the NIAID recommended that a study be undertaken which would determine the importance of histocompatibility matching.

At the present time, there are under contract six regional transplant centers involving 32 hospitals and a statistical data management center participating in the study. Plans are underway to add two additional centers representing four hospitals to the program. Data collection forms have been adopted, transplant registrars have been appointed and trained, and an instruction manual has been prepared for use by the registrars. A study protocol was prepared establishing objectives and design of the study, detailing the relationships of various participants in the study, and adopting standards for laboratory and clinical data.

All the 15 laboratories participating in the study have been tested for their proficiency in tissue typing and certified.

Committees and subcommittees of the participating investigators have been formed, and a Policy Advisory Board established to provide overall advice to the program.

Finally, the collaborative study became operational with the input of all transplants occurring after January 1, 1974.

The program will amass approximately 2,000 well-studied kidney transplants during 1974-1976. Two additional years of patient follow-up and one additional year of data analysis will be required with publication of the final report in Spring 1980. It is expected that this will be the definitive study on the significance of tissue matching in renal transplantation.

Bone marrow transplantation has been shown to be a promising treatment for a number of hematologic, neoplastic, and immunologic deficiency diseases. The key to clinical success lies in understanding and controlling the immunologic factors involved in rejection of the graft, or rejection of the host by the graft (GVH). Techniques presently under study include HL-A serotyping,family typing (genetic studies), mixed leukocyte culture, cell-mediated lympholysis, neutrophil agglutination, and mixed antiglobulin reactions. These procedures for donor recipient matching are evaluated.

III. Antilymphocyte Globulin Program

The Branch provides funds for a double-blinded study of Rabbit Anti-human Thymus Globulin (RAHTG). One hundred cadaver kidney transplant patients are treated with either RAHTG or a rabbit globulin placebo in a study designed to determine the efficacy of the RAHTG in preventing renal graft rejection. This RAHTG has been shown to be effective in prolonging skin or kidney grafts in subhuman primates. This is a joint project with the Naval Medical Research Program which developed the RAHTG and the University of California, San Francisco where the clinical trials are underway. The RAHTG being tested was developed with funds provided to the Navy by the Branch and animal testing was conducted with contract funds at Bionetics Laboratories. TIB funds provide for the collection and analysis of data and for the conduct of special immunologic tests to study patient immune responses. The absence of severe toxicity in any of the patients treated to date speaks for the safety of the products used in the study. Since this is a double-blinded study, no information concerning efficacy will be available until all patients have been entered. This phase of the study should be completed by September 1974. Patients will then be followed for a minimum of two years to determine long-term effect and toxicity. This study, which is the first to use the double-blinded randomized technique in a large number of patients, will serve to evaluate the predictability of the subhuman primate model in the selection of an active antilymphocyte preparation of human use. The use of a single institution with a standardized protocol limits the number of variables seen in many clinical studies.

IV. Regulation of Immune Responsiveness

During the past nine years, the Transplantation and Immunology Branch contracts program have been directed primarily toward drugs and histocompatibility matching as a means for preventing or overcoming graft rejection. It may be prevented by careful selection of the donor to avoid introducing a foreign antigen into the recipient. In actual practice, because of the multiplicity of antigens involved in histocompatibility, the chances of finding a perfect match when a cadaver donor is used are extremely poor. With the proliferation of organ exchanging programs and standardization of reagents through the NIH Tray Program, the numbers of four antigen matches for cadaver donor transplants, will hoepfully be increased. The problem of rejection in the mismatched transplants will remain.

In 1968 a study was started to evaluate new drugs with potential immunosuppressive activity. New techniques were developed and evaluated which were useful in testing humoral and cellular immune responses. These studies which were terminated during FY'74, demonstrated the activity of several of the new antitumor drugs.

Antilymphocyte serum or globulin has been claimed to be effective; the TIB controlled study now underway should help clarify the effectiveness of this biological immunosuppressant. Current drugs, while useful, are very toxic, producing marrow depression and therefore pan-immunosuppression. Chronic administration of high doses of steroids often produces the attendant complications of hypercortisonism.

In 1972, a meeting was held at Brook Lodge to review the state of the art of immunologic manipulation employed in various animal models to prevent the recipient of grafts from reacting to foreign histocompatibility antigens. These "immunological tricks" were applied prior to the onset of graft rejection and thereby attacked the problem of antigen recognition and suppression of cellular and humoral immune response to foreign substances. After reviewing the state of the art, the Transplantation and Immunology Committee recommended the establishment of a contract program to investigate new methods to regulate immune responsiveness. The Advisory Committee reviewed 48 responses to a sources sought announcement and recommended that 15 laboratories be asked to submit proposals. From the 13 responses received, six proposals were recommended for awards. These contractors will study the utility of a variety of procedures designed to alter humoral and cellular responsiveness.

V. Immunopathology Program

The Transplantation and Immunology Branch has had a contract with the Scripps Clinic and Research Foundation since 1968 to provide immunopathologic diagnostic facilities for transplant programs throughout the United States. Since the inception of this program, renal tissue and/or sera from nearly 2,700 patients have been submitted for study. During

the past year, 661 renal specimens and 576 serum samples from 704 patients were submitted by 65 contributing institutions. Immunofluorescent, radio-immunoassay, and antigen elution studies are used to detect anti-glomerulobasement membrane (GBM) tubular basement membrane (TBM) and, immune complexed antibodies, and the etiologic agents responsible for the various complexed antibodies, and the etiologic agents responsible for the various nephritides. These procedures have contributed significantly to the understanding of disease in many nephritis patients throughout the country. This contractor has been able to differentiate immunopathologically the mechanism of graft failure, i.e. whether failure is due to a recurrent nephritis, the deposition of circulating immune complexes in the renal glomeruli or tubules, or to an immunologic histo-incompatibility reaction. This has facilitated the diagnosis and treatment of patients with renal failure.

Current investigations in a rabbit transplant rejection model using eluted antigens from the glomeruli and tubules are intended to elucidate the role of these antigens in initiating nephritis and their mechanism of causing rejection. These basic studies are employed to acquire knowledge of the pathogenesis of kidney disease. These investigations should result in a better understanding of human kidney disease.

VI. Publications and Conference Support

As part of its information program, the Branch prepares an annual updating of the Catalog of Tissue Typing Antisera. The last issue of this Catalog was distributed to approximately 700 serum users in August 1973. The 900 page Catalog of over 1,300 sera in the Institute's Bank lists the availability of sera together with information submitted from 190 users. This includes reports of specificity, utility, titers and the frequency of reactions for various racial groups. This is the only such document available and is used throughout the world by transplantation immunologists, geneticists, hematologists, and scientists investigating the role of these antigens in a variety of diseases. The Manual of Tissue Typing Techniques revised during this fiscal year, contains current information on the various tests which are used in tissue typing and was distributed to investigators and used in tissue typing courses. The Branch also provides catalogs of other reagents including H-2 (mouse) antisera, complement components, and soluble antigens.

Contract funds provide partial support for the planning and conduct of the Fifth International Congress of the Transplantation Society which will be held in Israel in August 1974. Funds have been provided for a February meeting of surgeons and nephrologists in Columbus, Ohio to discuss techniques and the problems encountered in the pulsatile perfusion of cadaver kidneys. A workshop was held in April in St. Louis, Missouri where more than 300 laboratory workers discussed new techniques for typing and procedures for freezing viable lymphocytes to be used in histocompatibility typing.

INDIVIDUAL CONTRACT TABULATION

TRANSPLANTATION AND IMMUNOLOGY BRANCH
Contract and Agreements
Reagents and Techniques Program

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
AI-81298	Cleveland Clinic Foundation	Establish and operate a regional leucocyte phenotyping facility. To evaluate tissue typing techniques; match organ donor and recipient tissue by histocompability typing and cross matching techniques, and provide typing antisera to the NIH Serum Bank. Acquire, maintain, freeze and store a large reference panel of cells and serve as a quality control laboratory to determine the degree of reliance in the NIH Serum Tray program. The microcytotoxicity mixed lymphocytes culture (MLC), two way cross match, cell mediated cytotoxicity (CMC) and immune globulin techniques are applied to ascertain the degree of graft survival at this regional facility. They have quality controlled a respectable number of antisera and typing trays for the program.	06/28/68	$87,313	Renewal

INDIVIDUAL CONTRACT TABULATION

TRANSPLANTATION AND IMMUNOLOGY BRANCH
Contract and Agreements
Reagents and Techniques Program

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
AI-42508	University of Leiden	Search for anti-HL-A sera and improvement of histocompatibility testing techniques. To operate a large program to screen sera and to obtain and forward to the NIH quantities of sera found to contain histocompatibility antibodies. To compare and evaluate typing techniques. This program produces approximately 10,000 ml of much needed reagent quality sera for the program. A variety of new automated techniques are being compared. Information obtained will help in the standardization of procedures and materially improve the efficiency of histocompatibility typing.	06/24/65	$90,280	Renewal
AI-69-2071	Albert Einstein College of Medicine	Human neutrophil specific antibodies. To collect and quality control neutrophil specific antibodies and ascertain their import in graft survival. Neutrophil specific antigens appear to be a potential important	06/27/69	$44,000	Termination

INDIVIDUAL CONTRACT TABULATION

TRANSPLANTATION AND IMMUNOLOGY BRANCH
Contract and Agreements
Reagents and Techniques Program

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
	Albert Einstein College of Medicine	factor in bone marrow transplants. Approximately two liters of antisera were collected for the NIH Serum Bank.			
AI-80945	Irwin Memorial Blood Bank	Established and operate a regional leucocyte phenotyping facility. To provide prospective and retrospective histocompatibility typing and matching of organs for transplantation; collection of antisera for the NIH Serum Bank, and comparison of HL-A typing techniques. Matching for HL-A factors in kidney transplantation continues to show the unique superiority of HL-A identical sibling donors. Grafts from other related donors, in general, do not survive better than those from cadavers. Cadaver graft survival shows a borderline, but practically significant, correlation with HL-A matching; but more data is needed	03/27/68	$79,861	Termination

INDIVIDUAL CONTRACT TABULATION

TRANSPLANTATION AND IMMUNOLOGY BRANCH
Contract and Agreements
Reagents and Techniques Program

Contract/ Ag. No.	Contractor	Objectives and Findings	Date Initiated	Current Annual Level	Plans For FY 1975
	Irwin Memorial Blood Bank continued	especially with better matches. There appears to be a close correlation between the NIH micro-cytotoxicity test and the fluoro-chromasia techniques.			
AI-32508	Biomedical Research Institute, American Foundation for Biological Research	Freeze-drying of typing sera and processing, freezing and quality control of lymphocyte cells. To freeze-dry typing sera in a manner which completely preserves the typing reactivity of the reagent; in addition BRI is to develop a procedure to freeze, well-characterized lymphocytes in a multi-well tray which can be used as a uniform source of antigens. During FY'74 approximately 100 liters of antisera were freeze-dried in 1 ml vials. All bulk shipments are now sent at ambient temperatures. This has resulted in a reduction of the cost for an average U.S. shipment from $21.97 to $1.97. This laboratory has performed 2,270 micro-lymphocytotoxicity tests to determine the ability of the serum to withstand freeze-drying. BRI has	6/30/69	$113,858	Renewal

INDIVIDUAL CONTRACT TABULATION

TRANSPLANTATION AND IMMUNOLOGY BRANCH
Contract and Agreements
Reagents and Techniques Program

Contract/ Ag. No.	Contractor	Objectives and Findings	Date Initiated	Current Annual Level	Plans For FY 1975
	Biomedical Research Institute, American Foundation for Biological Research continued	prepared over 100 frozen cell trays from lymphocytes which have previously been frozen and thawed. The trays have been thawed and used in the microlymphocytotoxicity procedure with good results. A number of the trays have been sent out to another laboratory for testing.			
AI-22515	Flow Laboratories	Maintain a supply repository for processing and distribution of NIAID reagents and serum. To provide laboratory and warehouse facilities to process, store and ship reagents. This contractor made 1,275 shipments of bulk antisera and NIH tissue typing trays to 316 world-wide investigators. Forty-two liters of antisera were received and processed.	5/01/68	$130,730	Renewal

INDIVIDUAL CONTRACT TABULATION

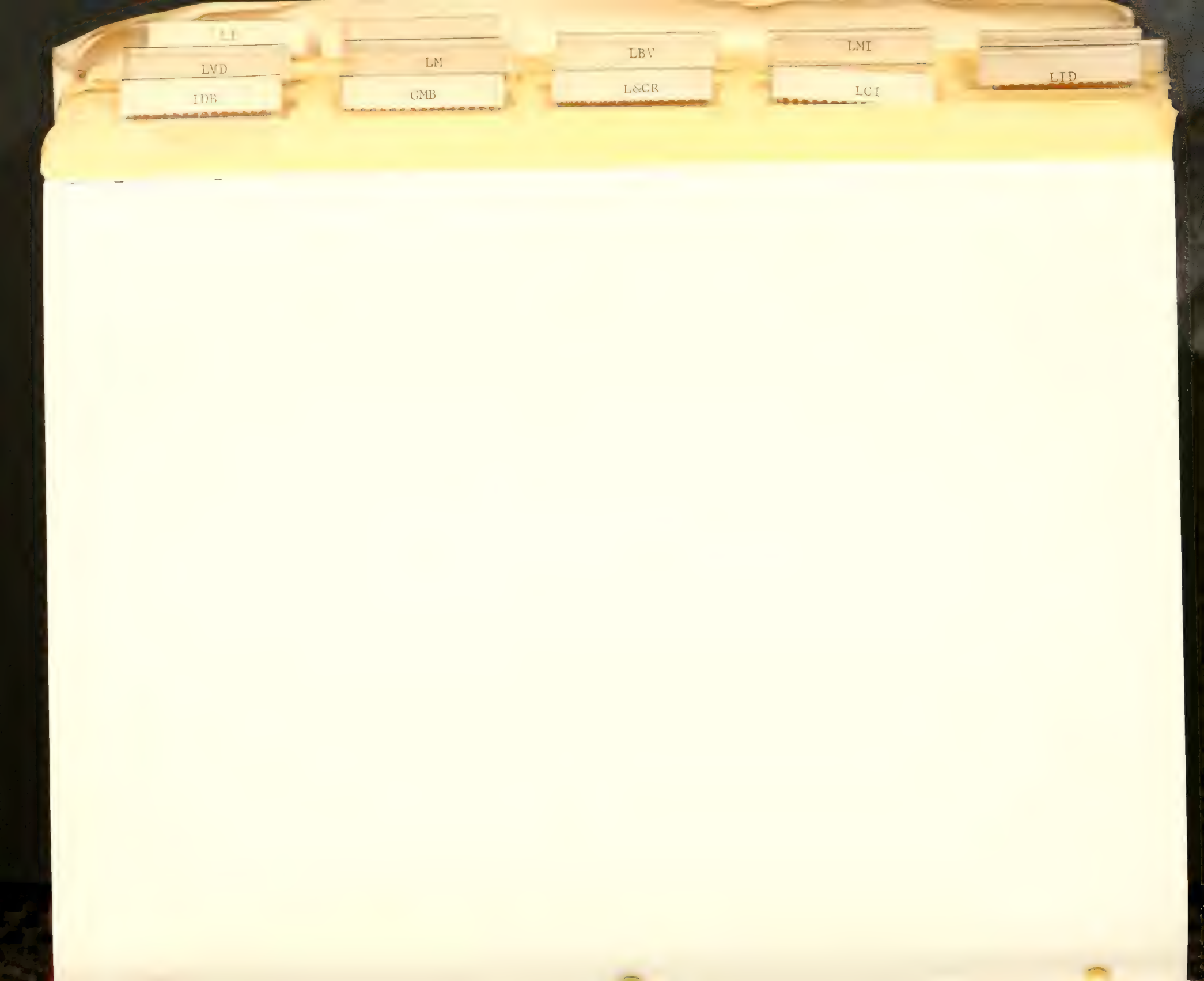
TRANSPLANTATION AND IMMUNOLOGY BRANCH
Contract and Agreements
Reagents and Techniques Program

Contract/ Ag. No.	Contractor	Objectives and Findings	Date Initiated	Current Annual Level	Plans For FY 1975
AI-12443	Microbiological Associates, Inc.	Preparation and evaluation of human histocompatibility typing trays. To produce and package for delivery, serum and cell typing trays for human histocompatibility typing techniques standardization. Approximately 50,000 serum typing trays have been used by 116 tissue immunologists in determing recipient and donor HL-A transplant antigens. The results of their typings are statistically comparable and aid in organ exchange programs and analysis of the efficacy of histocompatibility matching in organ graft survival.	6/21/71	$77,725	Renewal
AI-12257	Jackson Laboratory	Mouse alloantisera production, storage and distribution. To produce, store, quality control and distribute to qualified investigators, H-2 mouse alloantisera. At least 130 investigators have used these antisera for basic research in the immunogenetics of cellular and humoral immunity, graft rejection, tissue	1/02/68	$78,333	Renewal

INDIVIDUAL CONTRACT TABULATION

TRANSPLANTATION AND IMMUNOLOGY BRANCH
Contract and Agreements
Reagents and Techniques Program

Contract/ Ag. No.	Contractor	Objectives and Findings	Date Initiated	Current Annual Level	Plans For FY 1975
AI-12257	Jackson Laboratory continued	compatibility, tissue disease degradation and oncogenesis in the mouse model which is analogous to the HL-A tissue antigen system in humans.			


LVD
LDB
LM
GMB
LBV
L&CR
LMI
LCI
LID

INDIVIDUAL CONTRACT TABULATION

TRANSPLANTATION AND IMMUNOLOGY BRANCH
Contract and Agreements
Evaluation of Tissue Matching for Renal and Bone Marrow Transplantation Program

Contract/ Ag. No.	Contractor	Objectives & Findings			Date Initiated	Current Annual Level	Plans For FY 1975
	University of North Carolina School of Public Health continued	now operational with data collection forms, procedures, computer based data management system and operational policies developed. Records are being accrued on all transplants since January 1, 1974.					
		Evaluation of histocompatibility matching in renal transplantation. Provide clinical and laboratory data to statistical centers on kidney transplants. Maintain quality control of matching procedures, freeze and store cells and sera to allow subsequent immunologic studies. Data collection started on January 1, 1974.					
		# of Transplants	# of Hospitals	# of Laboratories			
AI-22536	Cleveland	40	1	1	6/29/72	$21,628	Renewal
AI-22529	Illinois	150	8	1	6/29/72	80,746	Renewal
AI-22530	Minnesota	125	3	1	6/29/72	79,509	Renewal
AI-22539	Rochester	70	7	4	6/29/72	45,824	Renewal
AI-22531	Texas	140	12	7	6/29/72	60,774	Renewal
AI-22537	Utah	20	2	1	6/29/72	20,404	Renewal

INDIVIDUAL CONTRACT TABULATION

TRANSPLANTATION AND IMMUNOLOGY BRANCH
Contract and Agreements
Antilymphocyte Globulin Program

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
AI-81327	Litton Bionetics Inc.	Primate preclinical Test Center for immunosuppression. To provide laboratory and clinical facilities and personnel to test and characterize antilymphocyte sera. The contractor has determined the ability of a variety of antilymphocyte sera or globulins to prolong skin and kidney graft survival in monkeys, baboons and chimpanzees. The data obtained from this contract has been used to pick the dose and treatment regimen and product to be used in the clinical trial now underway. During the course of this contract over 300 preparations obtained from a variety of sources were tested. With the completion of this preclinical testing the contract was terminated.	6/28/68	$212,000	Termination
AI-10001-05	National Naval Medical Center	Preparation and testing of ALG. To provide funds to collect and evaluate clinical and laboratory data on patients treated with antilymphocyte globulin.	11/1/71	$111,000	Renewal

INDIVIDUAL CONTRACT TABULATION

TRANSPLANTATION AND IMMUNOLOGY BRANCH
Contract and Agreements
Antilymphocyte Globulin Program

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
	University of California continued	gram developed ALG which is shown to be effective in prolonging grafts in sub-human primates, produces any significant improvement in the outcome of human cadaver kidney transplants. Under a Navy IND and protocols, the contractor is administering coded medications to patients. The Branch contract provides funds for reporting the results of this study and for the conduct of special in vitro tests to determine the patient's immunologic status during clinical trial. To date approximately 50 patients have been entered on study with an evidence of severe toxicity. The efficacy of the ALG will be evaluated upon completion of the study.			

INDIVIDUAL CONTRACT TABULATION

TRANSPLANTATION AND IMMUNOLOGY BRANCH
Contract and Agreements
Regulation of Immune Responses Program

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
AI-80949	University of Texas M.D. Anderson Hospital	Study the effects of chemotherapy on immunological responses of man and experimental animals. To inhibit or abolish the induction of primary and secondary antibody responses and stop ongoing antibody responses. Impair the development of, and abolish established, delayed hypersensitivity thereby preventing the rejection of skin homograft. Development of a number of new immunologic techniques to measure immune responsiveness has been accomplished. Evaluation of a number of immunosuppressive agents, l-asparaginase, l-glutaminase, cytosine arabinoside and aribonsyl 6 MP in animal models and subsequently in human patients was done but without any specific immunosuppressive effects.	6/21/68	$122,600	Termination

INDIVIDUAL CONTRACT TABULATION

TRANSPLANTATION AND IMMUNOLOGY BRANCH
Contract and Agreement
Immunopathology Program

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
AI-80621	Scripps Clinic & Research Foundation	Study of the etiologic events and antigen-antibody systems in human glomerulonephritis and the course of nephritis in transplanted patients. To provide an immunopathological analysis of human kidney diseases by assessing serum and renal tissue before, during, and after transplantation. This service contract is provided for all U.S. Kidney Transplant Centers. Currently 65 such centers have participated in this service and significant contributions concerning the etiology and treatment of renal failure and graft rejection have been made.	01/02/68	$168,399	Renewal
AI-80659	Medical College of Virginia	The procurement and characterization of post renal transplant rejection serum. To screen and develop techniques suitable to predict acute and chronic graft rejection in primary and multi-transplant patients and to collect for the NIH Serum Bank post renal transplant rejection	01/15/68	$52,302	Termination

INDIVIDUAL CONTRACT TABULATION

TRANSPLANTATION AND IMMUNOLOGY BRANCH
Contract and Agreement
Immunopathology Program

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
	Medical College of Virginia continued	serum. Significant results indicated that the anti-globulin test using donor kidney cells is the most sensitive test in cross-matching prior to kidney transplant and in detecting preformed circulating HL-A antibodies as well as early antibody formation which occurs after transplantation. There is an excellent correlation of post transplant antibody titers and the poor course of graft survival when using their test method.			

INDIVIDUAL CONTRACT TABULATION

TRANSPLANTATION AND IMMUNOLOGY BRANCH
Contract and Agreements
Publications and Conferences Program

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
Purchase Order	Ohio State University Hospital	Organize and conduct a Workshop Symposium for Organ Preservation. To provide facilities and personnel to plan and conduct a workshop on Pulsatile Perfusion. TIB funds were provided to conduct a two day meeting at Ohio State University. Approximately 200 surgeons and technologists discussed recent developments in the methodology of pulsatile perfusion. Items discussed included: Technican support for donor nephrectomy, transportation of kidneys and organization of regional sharing programs, characteristics of solutions, criteria for viability, physiologic changes in kidneys and medico-legal, ethical and moral problems in organ procurement.	10/16/73	$2,475	Termination
Purchase Order	Missouri-Illinois Regional Red Cross Blood Program	To organize and conduct a Tray Users Workshop. To provide facilities and personnel to plan and operate a workshop for histocompatibility	10/31/73	$2,390	Termination

INDIVIDUAL CONTRACT TABULATION

TRANSPLANTATION AND IMMUNOLOGY BRANCH
Contract and Agreements
Publications and Conferences Program

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
	Missouri-Illinois Regional Red Cross Blood Program continued	testing and lymphocyte cell freezing. TIB funds were provided to plan and conduct a 3 day meeting, attended by over 300 scientists and technicians to discuss new developments in tissue typing and to participate in demonstrations and bench practice in the various techniques for freezing viable lymphocytes. This meeting was designed to promote free exchange of information between laboratory personnel using the NIH supplied typing trays and other reagents. The aquisition of such information and experience is aimed at increasing the laboratory proficiency and improving the reliability of clinical typing.			

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A

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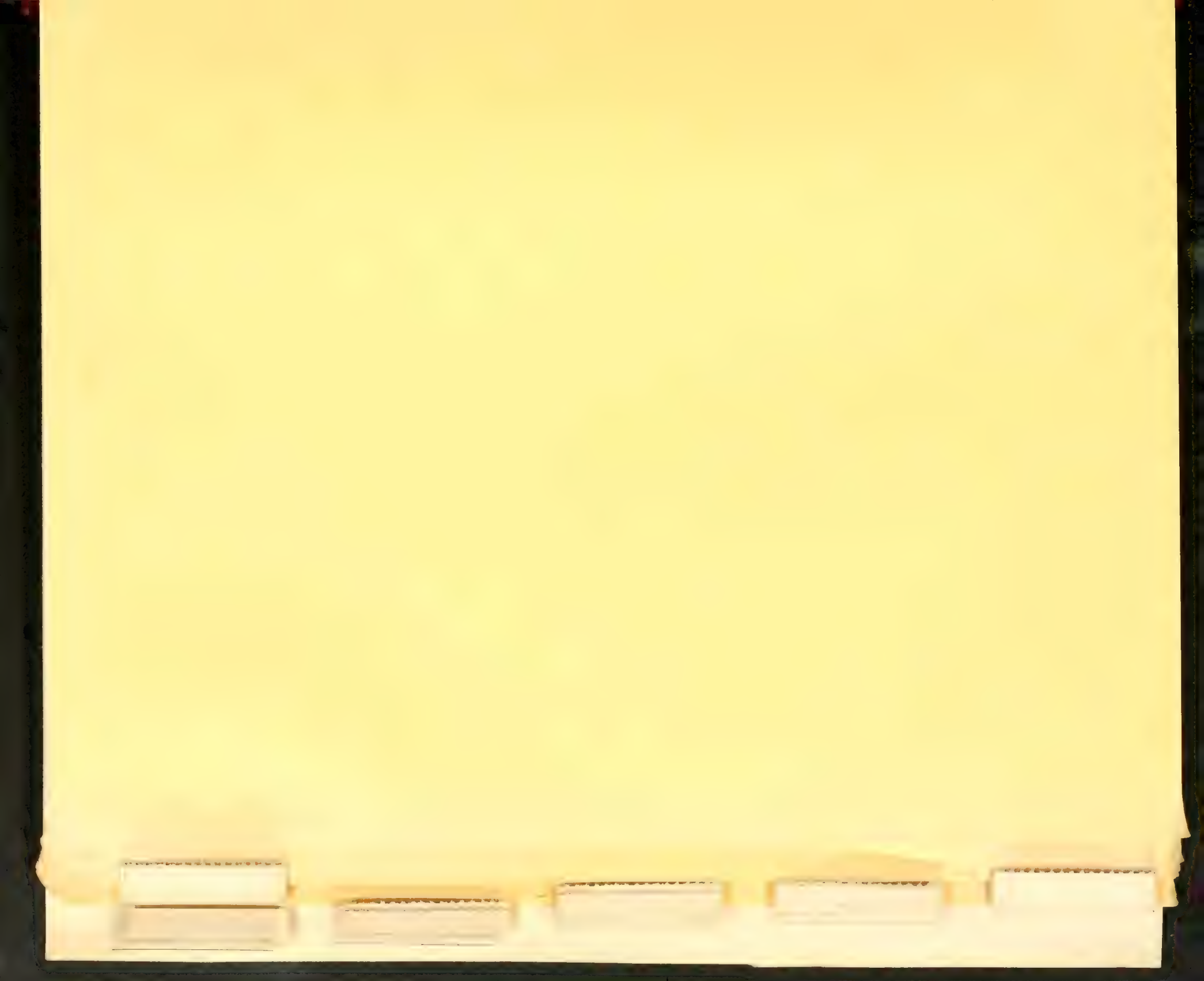
LVD
LM
LBV
LMI
IDB
GMB
L&CR
LCI
LID



INFECTIOUS DISEASE BRANCH
Program Status Report

The Infectious Disease Branch (IDB) actively supports research efforts in five program areas: Influenza, Hepatitis, Antiviral Substances, Bacterial Vaccines, and Viral Vaccines. In support of these activities, there are a number of general vaccine evaluation contracts which test potential vaccines from more than one of these program areas. These contracts are identified, with the proportion of effort and dollar allocation derived from each component program, in the individual contract tabulation tables.

VACCINE DEVELOPMENT AND VACCINE EVALUATION CENTERS

The goal of these programs is to develop vaccines for prevention of serious infectious diseases for which adequate vaccines are not now available. Program areas include influenza virus vaccines, other viral vaccines, and bacterial vaccines. Rutgers University is developing live virus mutants to respiratory syncytial virus (RSV) and parainfluenza virus types 1, 2 and 3 for use as vaccines. Flow Laboratories continues to prepare and safety test the vaccines developed by Laboratory of Infectious Diseases (LID) scientists and will also prepare and safety test virus mutants developed by Rutgers University.

The IDB has emphasized two kinds of vaccine evaluation centers: General study centers where the principal investigator has interest and expertise in the broad area of vaccine evaluation, and special study centers where the principal investigator has a primary interest in evaluating a particular type of vaccine. Included in our general study centers are the University of North Carolina, D. C. Children's Hospital, Baylor College of Medicine, Vanderbilt University, University of Colorado and the University of Rochester. Each of these contracts is performing studies under at least two of the vaccine program areas, and some of them are involved in all three vaccine program areas.

INFLUENZA PROGRAM

The establishment of a formal program on influenza within the Infectious Disease Branch of Collaborative Research began in FY 1974. Acting within the framework of recommendations from the Influenza Steering Group and the Infectious Disease Committee, the IDB began expanding research efforts on the viral disease that has been referred to as the "last plague of mankind". The long range goal of the Infectious Disease Branch Influenza Program is to support research activities leading to knowledge that will abort or lessen the impacts of future epidemics and pandemics. Areas of IDB research support are being closely coordinated with other agencies of the Government (particularly BoB, CDC, and Army) in order to avoid overlap or duplication of efforts. In addition, much of the current IDB program is directly related to the efforts of the intramural program in the development and evaluation of an attenuated vaccine.

Progress of the program during the past year includes the following:

LVD
LM
GMB
LBV
L&CR
LMI
LCI
LID

Workshops: The Influenza Steering Group held two workshops during FY 1974. The workshops assessed the current knowledge of animal influenza and influenza virus genetics. Information derived from these workshops will be used for program planning and identify possible areas for contractual support.

Vaccine Development and Evaluation: During the past year continued studies were conducted with the attenuated influenza A virus vaccine [Hong Kong (H3N2) ts-1-E], developed by the NIAID's Laboratory of Infectious Diseases. The vaccine was tested in various pediatric populations at Children's Hospital, D.C., Vanderbilt University, and the University of North Carolina (Phase I studies). The ts mutant appeared safe and immunogenic with no illnesses resulting from vaccination.

Because of the antigenic drift of the surface antigens of subsequent epidemic influenza A virus, the ts characteristic has been transferred to A/England/72 (H3N2). Vaccines of various clones of the ts-1-E recombinants were prepared by Flow Laboratories. Trials conducted with normal adults at the University of Rochester indicated that one clone was underattenuated and one clone was overattenuated. These studies strongly indicate that the ts lesions are polygenic and that various degrees of virulence will accompany the ts recombinants. Further evaluation of the London recombinants will be conducted in young adult volunteers at the University of Virginia and of Rochester University.

In order to learn the nature of the ts lesions of the various attenuated influenza A mutants and recombinants, Dr. Robert Simpson, Rutgers University, was awarded a contract to investigate and define the genetic and biochemical defects of the viruses. In addition to sponsoring the study on genetic analysis of the viruses, the IDB is in the process of awarding a contract for development of an *in vitro* assay for degrees of attenuation for influenza viruses. There is evidence that ciliary activity of tracheal organ cultures can be correlated with degrees of virulence of mutagenized or recombinant influenza viruses. Such an assay would be invaluable for selection of future live vaccine recombinants as antigenic drift or shift dictate production of recombinants with different surface antigens.

An experimental inactivated influenza vaccine (monospecific for the neuraminidase surface antigen) is currently being evaluated in phase I challenge studies at Baylor University. The concept of "infection permissive" vaccines for long lasting immunity is being actively investigated by the IDB influenza program, since preliminary results need further substantiation.

Pediatric populations are not considered "high risk" populations for influenza, but the morbidity among school children is very high. To this end, it would be desirable to break the transmission chain of influenza by inducing immunity in this population. The current inactivated influenza vaccines are not recommended for children because of systemic reactogenicity. There is, however, evidence that the various components of the influenza virus retain antigenicity and immunogenicity when properly isolated. Dr. Robert Webster, St. Jude Children's Hospital, is currently investigating the feasibility of developing a protein subunit vaccine for pediatric populations. This research has just been initiated and it is expected to take several years before

evaluation of this approach can occur.

Immunology: Various aspects of the immunological response to live and inactivated influenza vaccines are being conducted at the Baylor College of Medicine, the University of Virginia, and the University of Rochester. There is a paucity of definitive data on protective mechanisms from influenza infection, and these contractors are attempting to define the host's response to this agent.

There are numerous questions which remain to be answered on the pathogenesis, epidemiologic, and immunologic aspects of influenza. The IDB is in the process of establishing an Influenza Research Center which would provide expertise in these areas and seek answers to these questions. In the event of a pandemic in the late nineteen-seventies, the data accumulated from such a center should contribute to a better understanding of the disease and methods for protecting against or controlling future pandemics. The complementation of the research center and studies conducted at the general vaccine evaluation centers provide a firm foundation for a coordinated effort in influenza research.

HEPATITIS PROGRAM

The goal of the hepatitis program is to support research activities leading to the control of hepatitis A and B infections. Major emphasis is being placed on studies of hepatitis B infection since recent knowledge of hepatitis B antigen (HB_sAg) and antibody (anti-HB_s) characteristics has made it possible to better define this disease. The projects supported were initiated after careful Institute planning, which included a series of workshops on hepatitis B subtypes, animal models, immune complexes and hepatitis, immunization and epidemiology. The recent demonstration of the causative agent of hepatitis A by immunoelectronmicroscopy in infectious material has produced an impetus for studies with this virus, and several preliminary experiments involving this agent will also be integrated into this progress report.

Epidemiology: In order to help identify "high risk" populations for future passive or active immunization trials, the IDB is supporting two epidemiological studies. The first is a collaborative effort with the Center for Disease Control (CDC) to determine to what degree hemodialysis patients and staff, and family contacts of these patients can be classified as "high risk". These studies (involving two separate hemodialysis units) include identification of infecting source, method of spread, prevalence and incidence of infection and illness, and incubation period of the disease. To date, the data represent a point prevalence study of patients and dialysis assistants from March 1973 - February 1974, and demonstrate that a marked difference in both previous hepatitis B infection and continuous exposure to hepatitis B infection for all categories of study participants seems to exist in Atlanta as contrasted with Mississippi. The great majority of these hepatitis B infections appear to be unassociated with evidence of clinical or subclinical illness and appear to be associated with exposures which occur in the dialysis unit setting, primarily involving contact with blood and dialysis equipment. A partial explanation for the greater association of hepatitis B infection with the Atlanta unit may be the continuing large exposure to

hepatitis B antigen on that unit in the form of a number of persistently antigen positive renal dialysis patients.

The point prevalence study of patients, staff, and household members at the time of enrollment failed to show any relationship between evidence of hepatitis infection and age, sex distribution, previous blood transfusions, presence of shunt or fistula, history of hepatitis, etc. However, such factors as race (patients with previous evidence of hepatitis B infection were more likely to be black), length of hemodialysis before entering the study, assisting patients with dialysis and duration of contact with hemodialysis patients all seem to contribute to the risk of hepatitis B infection.

The frequency of prior contact with HB_sAg positive patients and staff as a risk factor for development of hepatitis B infection suggests a positive correlation; i.e., the more exposure to potentially infectious patients and staff, the greater the risk of HB_sAg infection.

This study has not been in progress long enough to establish the risk of hepatitis associated with such factors within the unit such as sharing by patients of dialysis equipment, environmental sanitation, technique and hygenic habits of staff, etc.

The second epidemiologic study supported by the NIAID is being conducted at West Seneca State School, Buffalo, New York. Although this contract was awarded in June 1973, actual investigations were begun in September 1973. Initial screening for HB_sAg and anti-HB_s in approximately 1,000 residents and staff members indicated that 10% of the residents were HB_sAg positive and 44% were positive for anti-HB_s. The prevalence rate of anti-HB_s in school staff was 24% and no staff members were positive for HB_sAg. These observations suggest a high prevalence of prior exposure to HB_sAg in this population. However, it should be pointed out that a significant proportion of the population (48% of residents and 74% of staff) have no serologic evidence of infection as demonstrated by the absence of HB_sAg and anti-HB_s activity in the serum. The acquisition of hepatitis B infection appeared to be related to a number of factors, in particular, to the duration of residence at the institution. Close surveillance is being maintained on new admissions to the school, but the study has not been in progress long enough to determine the incidence of infection.

Passive Immunization: In FY 1973 the NIAID, in collaboration with the Veterans Administration (VA) hospitals, initiated studies to determine if high-titered anti-HB_s gamma globulin can prevent or modify hepatitis B infection in persons experiencing accidental parenteral exposure to this disease (Needlestick). Normal gamma globulin preparations have little or no effect in preventing icteric hepatitis B infection. Preliminary evidence from other studies suggests that gamma globulin containing anti-HB_s might be effective in preventing hepatitis B. To date 224 subjects have been randomized into the study. Based on an anticipated incidence of 30% of icteric hepatitis in the control group, 534 subjects would have to be randomized into the study to yield meaningful data. However, only five cases of HB_sAg positive icteric hepatitis have been observed thus far, suggesting that risks

from parenteral exposures are not as severe as previously suspected. This is a double-blind study, however, but the Operations Committee of the VA Hepatitis Cooperative Studies periodically review the data (not blinded) and have recommended continuation of subject randomization indicating that useful data are still being accumulated.

The NIAID is also collaborating with the VA Hospitals on a post-transfusion study (PTS II), administering either conventional low-titer or high-titer anti-HB_S to determine if these preparations lower the incidence of hepatitis B infections. It was anticipated that 1,000 entries into this study would be required, and 518 have been randomized to date. The randomization rate is as expected, so no difficulties are anticipated in obtaining significant data in the reference time frame. This is also a double-blind study so no results are currently available.

The gamma globulin preparations for the above studies were procured by the NIAID from Cutter Laboratories.

Animal Models: Progress is continuing in characterizing the agent of hepatitis B in animal models. The general susceptibility of primates to hepatitis B, as recognized by antibody to HB_SAg, appears to parallel the phylogenetic scale, with those primates closest to man being the most susceptible, notably the chimpanzee and the rhesus monkey.

Further studies with hepatitis B (subtype adw) adapted to rhesus monkeys have suggested that the infectious agent is not as stable as previously suggested. Infectivity of agent for rhesus monkeys was (eliminated) by 100, 80, 60, and 50 C for 1 hour, but the agent was stable at 37 C for 1 hour. The virus was inactivated by lipid solvents, detergents and acids. Formaldehyde (1%, 1 hour), Wescodyne (1%, 1 hour) and Chlorox (1%, 1 hour) destroyed infectivity of the virus, but Roccal (1%, 1 hour) did not inactivate HBV for rhesus monkeys.

Pathogenesis studies of hepatitis B in chimpanzees has been a collaborative effort of the LID, IDB, CDC, and FDA. As a result of these studies, the chimpanzee has been shown to respond to hepatitis B virus infection virtually identically the same as man. For example, incubation period for the disease varies from 19-64 days before detection of HB_SAg, liver function tests (SGPT and SGOT) become elevated, and anti-HB_S develops.

Pedigreed pools of infectious HBV have been derived from chimpanzees and will serve as challenge pools for future active immunization tests.

Since data derived from animal models thus far suggest the antibodies to the coat protein (HB_SAg) of the HBV are protective, an inactivated experimental vaccine has been prepared (subtype ayw) and has been inoculated into chimpanzees at the Holloman Chimpanzee Colony. After appropriate serological monitoring for six months, the chimpanzees will be challenged with pedigreed HBV to determine efficacy of the vaccine.

The IDB is currently procuring 200 liters of HB_SAg-rich plasma for future use in potential vaccine preparations. The various components present in

the plasma (coat, core, DANE particle) will be separated out and evaluated as possible immunogens. The separations will be performed by the MAN Laboratory (RRB contract). Future trials of potential vaccine candidates will be conducted in chimpanzees for safety, antigenicity, and efficacy before considering use of such immunogens in man.

Because the marmoset (Saguinus mystax) has been shown to be susceptible to hepatitis A infection, the NIAID, CDC, and FDA are cooperating to establish and maintain a marmoset colony. Such a colony will ensure small animals for future hepatitis A studies. Preliminary experiments recently conducted have suggested that the chimpanzee can be infected with hepatitis A and will serve as an animal model for this virus.

Immunohistochemical Assays: Currently one of the most sensitive techniques for the detection of HBV antigens in tissue consists of immunohistochemical assay procedures. In FY '73 the NIAID established a contract for applying immunohistochemical assay techniques to the detection of hepatitis B antigens, particularly in primate specimens from animals involved in hepatitis infections. Results from these studies have contributed greatly to the concept that the DANE particle is indeed the virion of hepatitis B. By using highly specific reagents, Dr. Edgington, Scripps Clinic and Research Foundation, has shown that the core particle is produced in the nucleus of the hepatocytes and that the coat antigens are synthesized in the cytoplasm. The complete DANE particle is found in the hepatocyte nucleus, cytoplasm and plasma.

Testing Laboratory: The NIAID currently supports the Hepatitis Testing Laboratory at the American Red Cross. The laboratory's function is to test for hepatitis antigens and antibodies by the most sensitive techniques available. Tests available include subtype identification, radioimmunoassay procedures for antigen and antibody, and passive hemagglutination tests. During the past year, the laboratory has performed hepatitis B antigen and antibody tests for the NIAID hepatitis programs, and also for the hepatitis programs of other NIH Institutes and the Food and Drug Administration. An additional function of the laboratory is to aid in the standardization of methods for detecting hepatitis antigens and antibodies.

ANTIVIRAL SUBSTANCES PROGRAM

There are many viral diseases for which there is no prophylaxis or therapy presently available and which constitute serious public health problems. There is, therefore, a need for the development of antiviral substances for the treatment of these diseases. Planning for a program to help meet this need began in July 1969. At that time a thorough study of the field revealed that there was considerable increasing interest in this area and that interferon or interferon inducers were the most promising antivirals to exploit. This continues to be the case, although more recently there have been a number of case histories published which indicate that adenine arabinoside (Ara-A) and iododeoxyuridine (IDU) have been effective against herpes encephalitis and other serious viral diseases. The program continues to be alert to new developments emanating from scientist-originated basic research so as to properly provide the needed stimulus to hasten the field by applying

this knowledge to practical use in man. The general intent of the program is to serve as a focus for all activities on antivirals conducted by Extramural, Intramural and Collaborative Research of the National Institute of Allergy and Infectious Diseases and to some extent, other Institutes of the National Institutes of Health.

Following the advice of ad hoc committees and the Infectious Disease Committee, there has been a gradual shift of emphasis toward more clinical studies, as some of the original goals in basic studies to fill some of the gap areas are accomplished. There are also increased efforts in the area of antivirals other than interferon, as witness studies on Ara-A and IDU. The studies currently being pursued include the following:

Interferon Assay: In order to support and expedite research in the area of interferon, studies were undertaken to develop more rapid quantitative standardized assays. A variety of parameters involved in the assay systems were studied, and the information obtained did lead to considerable reduction in the time required for the performance of these assays. A rapid assay system utilizing the cytopathogenic effect (CPE) in plastic trays with several wells has been developed. This new assay quantitates the interferon in the test solution by measuring the resultant neuraminidase yield-reduction of the viral challenge. This system can be modified and used to measure all types of interferon. During this last contract year this contractor (Medical College of Wisconsin) has concentrated on simplifying the technique so that all manipulations for the assay can be done in the same tube, from cell seeding to final spectrophotometric reading. This is possible because of the new synthetic neuraminidase substrate (methoxyphenol-neuramic acid) provided by the Research Resources Branch. This simplified, rapid and sensitive assay will provide a needed common assay for all interferon investigators so that results can be more readily compared.

Standards and Reagents: In collaboration with the Research Resources Branch, the ASP has successfully established reference reagents for rabbit interferon, mouse interferon, and interferon inducers [polyinosinic· polycytidylic acid (poly I·poly C)]. The two interferon preparations (rabbit and mouse) were originally prepared in the wet frozen state. The ASP has now made available lyophilized preparations of mouse interferon and also human interferon. These reagents have undergone standardization analyses and unitage assigned which properly corresponds to the original materials established as the standards. A new contract has been awarded so that the remaining wet frozen interferon (rabbit) can be prepared in the lyophilized state. This procedure requires some innovative techniques since all interferons have proven difficult materials to work with in respect to their stability and resistance to manipulation. A separate contract to produce highly specific antisera to mouse and human interferon is continuing. Antiserum to mouse interferon has been produced in rabbits and distributed to various workers in the field. Use of this reagent has helped to prove that there are two types of interferon produced in the mouse model. Use of this antiserum has also aided in developing purification methods for interferon using affinity chromatography. Work on the production of specific antisera to human interferon should yield a product ready for distribution by the end of this contract year. An important by-product of this contract has been the identification of the multiple

LVD
CMB
LM
LNCR
LBV
LCI
LMI
LIP

types of interferon, e.g., antisera developed to human diploid cell interferon and to human leucocyte interferon indicate that the two interferons have antigenic differences.

Purification: If exogenous interferon is to be used in man, there is a need for purification and concentration. The ultimate goal would be purification to the extent that the chemical structure can be defined and eventually interferon manufactured synthetically. Currently studies on purification of human interferon are being conducted through a collaborative effort involving NIAMDD and both intramural and collaborative components of NIAID. The use of affinity chromatography techniques utilizing specific interferon antisera has proven very useful in these studies. A new contract has been initiated to determine the most effective way of maintaining the stability of purified interferon. Purification studies have indicated that these studies are needed since the interferon loses activity with purification.

Production: If interferon proves effective in the treatment of human viral diseases, two methods of administration are available: the administration of exogenous interferon, produced in vitro, or endogenous interferon through the use of inducers. For the former there is a need for the development of a technique which will provide a more efficient, safe and economically feasible method of producing large quantities of human interferon. A wide variety of cells have been tested to determine their relative capacity to produce interferon using a series of inducers. There is very little difference between cells in their ability to produce interferon, but human amnion cells and foreskin fibroblast diploid cells are the most promising. Two contracts are supported in this area, one to develop better production methodologies, including superinduction (NYU). This contract continues to be very productive. They also provide crude human diploid cell interferon for purification studies. A second contract (Lee Mac) is for the application of these techniques to large scale production utilizing a unique cytogenerator. Success of the production contracts is indicated in the interests expressed by commercial manufacturers who are contemplating production. The program is also supporting work in the evaluation of interferon inducers which may have clinical potential; these include biological inducers and poly I·poly C lysine complex.

Animal Models: As part of the effort to develop potential antivirals for clinical use, a series of animal-viral disease models, which closely mimic diseases in man, were established. This constitutes the Cooperative Antiviral Testing Group of the ASP. The models selected encompass as wide a range of animal species and viruses as possible with the exception of the non-human primate. Utilizing these models, comparative in vivo evaluation of various antivirals which show some promise as prophylactic and/or therapeutic agents is possible. The mode and dose of infection have been selected so as to approximate the natural mode of infection as much as possible. Baseline data continue to be accumulated using interferon (exogenous and endogenous), and this will then serve as the reference for comparison of new antivirals as they develop. If any one of these compounds proves to be efficacious and non-toxic, human studies can be considered.

Clinical Studies: The state of the art has progressed to the point where some clinical trials are now underway and several others could be initiated. This is occasioned by better production methodologies for exogenous interferon; sufficient studies to show high levels of exogenous human interferon are not toxic, and also the development of a more effective inducer (poly I·poly C lysine complex). A study to evaluate poly I.poly C against herpes keratoconjunctivitis has been completed and the data currently being evaluated.

Exogenous human leucocyte interferon is being used in prophylactic studies for the prevention of the recurrence of herpetic keratoconjunctivitis in patients who have a history of such a disease. It has been shown that individuals who have had two episodes of herpetic keratoconjunctivitis have a 40% chance of having a recurrence within the next two-year period. Exogenous interferon is also being studied in the treatment of disseminated herpes zoster, which is a major problem in immunologically compromised patients. These studies were selected for support because it is anticipated that interferon will have its most beneficial effect in disease states that are dependent on de novo viral replication for their pathogenesis. Animal studies have indicated that interferon has a definite role in post-exposure treatment of rabies. The results are sufficiently encouraging to encourage clinical evaluation.

Preliminary studies in serious viral infections such as herpes encephalitis have suggested that IDU and Ara-A may have definite beneficial results. A study at the University of Alabama has therefore been developed to test, in a comparative fashion, IDU, Ara-A and placebo to determine whether both or either of these compounds does indeed have clinical efficacy. Because Ara-A has been shown to be nontoxic in man, it is also being studied for a beneficial effect against disseminated herpes zoster and herpes virus hominis in the immunologically compromised patient where both of these viruses present serious problems. It is hoped that Ara-A will also eventually be shown to be beneficial against neonatal herpes and cytomegalovirus infections, which continue to be problems with the newborn.

BACTERIAL VACCINE PROGRAM

Bacterial Meningitis Vaccines: Two organisms (Hemophilus influenzae and Neisseria meningitidis) are primarily responsible for bacterial meningitis in young children and infants. There is epidemiological evidence that about 40 cases of serious infections with H. influenzae per 100,000 and 20 cases of meningococcal C meningitis per 100,000 occur in children between the ages of three months and six years. Infants three months through two years of age are at highest risk of serious infection. Effective immunoprophylaxis would be of great benefit to this segment of the pediatric population.

A candidate H. influenzae type b polyribose phosphate (PRP) vaccine has been developed by Drs. Porter Anderson and David Smith at the Children's Hospital Medical Center in Boston. This group, as well as the contractors at the University of Southern California, the University of Colorado, and the University of Rochester, are performing clinical evaluation studies of PRP

in children. Children's Hospital, Boston, serves as a serological reference laboratory performing radioimmunoassays for anti-PRP antibodies in most sera obtained in these studies. The results of clinical studies to date indicate that PRP is immunogenic in children over the age of 18 - 24 months but that there is very little response in a substantial portion of infants under one year of age. The significance of this low level antibody response in infants with regard to protection is unknown, but many of the investigators in this field feel that the low level may be protective and that an adequate efficacy trial of the PRP vaccine should be conducted.

Clinical studies in children of the groups A and C meningococcal polysaccharide vaccines developed by the Walter Reed Army Institute of Research are being carried out by contractors at the University of Connecticut, the University of Colorado, and the University of Southern California. The contract with Rockefeller University supports a reference testing laboratory to measure, via the radioimmunoassay, the antibody response in sera from infants and children immunized with groups A and C meningococcal polysaccharide vaccines by all the IDB contractors conducting clinical evaluation of these antigens.

In general, results from clinical studies indicate that the quantitative ability of infants to produce antibodies to the meningococcal groups A and C polysaccharides is a function of age. Three month old infants respond weakly but consistently to the group C antigen and weakly and inconsistently to the group A antigen. Older infants (7 - 18 months) respond consistently to both antigens.

Results obtained by the University of Connecticut group indicate that booster injections of group C polysaccharide given at 7, 12, or 18 months to infants who had received a primary injection at three months result in decreased antibody production, as compared to a single injection given at a later time. The A vaccine behaves differently, and a booster can be demonstrated at seven months and one year among children primarily immunized at three months. This suggests that there is more exposure of infants to cross-reacting antigens of A than C during the early months of life.

Both meningococcal vaccines appear to be well tolerated by infants with very few adverse local or systemic reactions.

A meeting of the meningitis vaccine contractors was held in conjunction with the winter meeting (January 21-22, 1974) of the Infectious Disease Committee and the results of meningococcal and H. influenzae vaccine clinical studies were presented. The consensus of the group was that clinical studies were nearing completion and that the next step should be a large scale trial to determine the efficacy of the vaccines in preventing bacterial meningitis in children and an efficacy trial of H. influenzae and meningococcal C vaccines has been recommended by the Infectious Disease Committee.

Pneumococcal Vaccine: Monovalent and polyvalent vaccines of pneumococcal capsular polysaccharides have been developed and clinical evaluation studies conducted. Most of the work on vaccine development and the epidemiology

of pneumococcal infections has been done through a contract with the University of Pennsylvania. A vaccine trial using two hexavalent vaccines was initiated in January 1973 at Dorothea Dix Hospital, Raleigh, North Carolina, and data are now available for 823 of the 1,081 participants in the study. Approximately 64% of the participants are 50 years of age or older. Rather minor adverse reactions were observed; and, as a group, the vaccinees responded well to each of the 12 antigens. These vaccinees will be followed for development of pneumococcal infection. To date, of the six deaths attributed to pneumonia, three were in the vaccinees and three were in recipients of placebo. There has been a decrease in the number of bacteremic infections in this population during the past two - three years, which may affect the results of this efficacy trial. The Pennsylvania contract continues to serve as a reference serological laboratory using the radioimmunoassay to measure type-specific antibody response to pneumococcal vaccine. The Principal Investigator, Dr. Robert Austrian, also continues to render scientific-technical assistance to other pneumococcal vaccine contractors.

A large scale trial is underway to determine the efficacy of a dodecavalent pneumococcal vaccine in preventing type-specific pneumococcal pneumonia and its impact on pneumococcal pneumonia and total respiratory disease in adults over age 45. The study is being done in San Francisco under a contract with Kaiser Foundation Research Institute. Immunizations began in January 1974, and to date, approximately 9,000 people have received injections (including both vaccine and placebo). Vaccinations will continue until 10,000 to 15,000 people are in the study. So far, no serious reactions to the vaccine have been reported.

An octavalent pneumococcal polysaccharide vaccine is being evaluated in infants and children in clinical studies at the University of California, San Francisco. The studies have indicated that immunization of infants greater than six months of age with octavalent vaccine can be accomplished with safety and will result in significant antibody response. Infants less than six months of age appear, in preliminary studies, to respond less well to the pneumococcal vaccine than the older children.

The pneumococcal vaccines used in all of the above mentioned studies were produced under a contract to Eli Lilly & Co. which will expire in June 1974.

Acute otitis media is a very important childhood disease and affects populations of all racial and social classes. Almost every child has otitis media at least once and 20 to 30 percent have six or more such occurrences. It is well established that pneumococci are responsible for 40 to 50 percent of the episodes of acute otitis media.

Acting on the recommendation of the Infectious Disease Committee, the IDB staff is preparing to initiate (before the end of FY '74) two or three contracts to study the efficacy of the octavalent pneumococcal vaccine for the prevention of acute otitis media in infants and children.

Streptococcal M-Protein Vaccine: Group A streptococcal infections play a major part in septic pharyngitis and skin infections as well as an antecedent role in rheumatic fever and acute glomerulonephritis. An effective multivalent vaccine containing at least six of the predominant serotypes of M-protein would make a major impact on reducing the prevalence of these serious illnesses.

The contract with the University of Chicago has led to the development of several satisfactory candidate M-protein antigens which currently are being evaluated in adult volunteers for safety and immunogenicity. The objective of this study is to demonstrate whether a vaccine composed of M-protein is efficacious against disease. Adult volunteers have been immunized intranasally with soluble type 1 M-protein and challenged with live streptococci. In contrast to previous results with parenteral immunization (in which most of the subjects exhibited significant antibody responses) intranasal immunization with M-protein evoked very poor antibody responses in both the sera and nasal washings. However, despite the lack of detectable antibody, the intranasal route of immunization clearly prevented the subsequent colonization of the pharynx with the challenge organism. Significantly less illness and positive throat cultures were seen in vaccinees than in controls. Preliminary data have been obtained on immunization of adults with the M-protein type 12 vaccine, followed by challenge. Again, volunteers immunized with the type 12 M-protein by the intranasal route had no significant increases in antibody titer but were apparently protected. Parenteral immunization with type 12 protein also conferred protection. These preliminary data are encouraging and studies are currently under way with additional volunteers. Similar challenge studies are beginning with type 3 M-protein vaccine.

In order to compare various methodologies for preparing pure streptococcal group A M-protein antigens, Abbott Laboratories was contracted to produce 2 grams of crude M-protein each of serotypes 1, 6, and 12. Four laboratories actively engaged in streptococcus research have agreed to purify aliquots of the crude M-protein by their own methods and perform comparative tests to determine the method for obtaining the most pure and immunogenic antigens. A recent planning session was held with the collaborators. The crude material will be delivered to them by Abbott Laboratories in May 1974, and it is anticipated that the purified antigens will be ready for testing in January 1975. The IDB is assisting the collaboration further by supplying a small amount of funds for the purchase of supplies.

The results of this collaborative effort and the results obtained in the University of Chicago studies will be presented to the NIAID Infectious Disease Committee. They will be asked to use these data to evaluate the potential of a vaccine against streptococcal disease.

Mycoplasma pneumoniae vaccine: Two large lots of formalin inactivated Mycoplasma pneumoniae vaccine (OSU-1A and OSU-1B) have been produced by Ohio State University and have been tested for safety and potency by Ohio State and by Dr. Senterfit at Cornell University. The Ohio State contract will terminate at the end of FY '74; however, the Cornell contract has been

renewed to serve as a serological testing laboratory for the assay of sera from the University of Virginia/Parris Island efficacy trial (see below). Five thousand doses of placebo (phosphate buffered saline with formalin) are being purchased by IDB from Ben Venue Laboratories.

A new contract with the University of Virginia is being initiated specifically to study the clinical acceptability, antibody response, and efficacy of the inactivated M. pneumoniae vaccine (OSU-1). The OSU-1 vaccine has (at the present time) been tested in more than 50 people. Studies performed under another contract with the University of Virginia have shown it to be sufficiently antigenic but some side effects were observed. Under this new contract, Dr. Wenzel, the Principal Investigator, will look for acceptability and serum and nasal antibody responses in 20 volunteers at the University of Virginia. Following this, approximately 150 incoming recruits at Parris Island, South Carolina, will be vaccinated and observed closely for side effects and antibody responses. Assuming that the vaccine is acceptable, an efficacy study in 7,500 volunteers will begin. This final phase will hopefully get underway in July of 1974.

Under a contract with Baylor University, studies are being conducted to develop an experimental challenge system for M. pneumoniae infections. Administration of tissue-culture grown M. pneumoniae by small particle aerosal inoculation appears to produce lower respiratory disease comparable to that seen naturally. Preliminary data using this system indicate that the OSU-1 vaccine offers some protection to the M. pneumoniae challenge.

Venereal Disease: Venereal disease is the major infectious disease public health problem in the U. S. At an ad hoc meeting of experts in this field sponsored by the IDB in July 1973, a number of projects appropriate for the contract mechanism were recommended. These are projects which hopefully would contribute to resolving this problem.

Plans are underway for initiating a venereal disease program by awarding one or two contracts before the end of FY '74 for the in vitro cultivation of Treponema pallidum, the causative organism of syphilis. Cultivation of T. pallidum in an in vitro system is considered of paramount importance for research on this venereal disease and for the possible development of candidate immunizing agents. Stimulation of basic research effort in this area by the contract mechanism will hopefully shorten the time until a potential vaccine can be made available.

VIRAL VACCINE PROGRAM

Respiratory Syncytial Virus (RSV) - Parainfluenza Viruses: Respiratory syncytial and parainfluenza viruses are the principal causes of severe lower respiratory tract disease among infants and children. The need for effective vaccines to reduce the morbidity and economic toll of these pediatric viral illnesses remains apparent. A temperature-sensitive (ts) mutant of respiratory syncytial virus, developed by the Laboratory of Infectious Diseases, NIAID, has been evaluated in infants of one year of age. During the past year lower doses of the RSV ts-1 vaccine were tested. It was hoped that the lower dose of vaccine would result in fewer adverse reactions; for example

less rhinorrhea. However, if the child were infected by the vaccine virus, he was just as apt to develop rhinorrhea whether he received the low or high dose of vaccine. Because the ts-1 mutant did revert to phenotypically wild-type virus and spread to susceptible contact controls in a few instances, it was decided that no additional evaluation of it would be performed. At the present time epidemiological data on RSV infections and illness are being obtained.

Dr. Simpson at Rutgers has developed double mutants to RSV. Some of them will be used as seeds for preparation of vaccines during the coming year. He has just begun work on the isolation of double mutants to parainfluenza virus types 1, 2, and 3, so none of them will be available for use as vaccine seeds this year.

Additional work on the purified concentrated lots of monovalent and trivalent inactivated parainfluenza virus vaccines showed an inconsistency in the pattern of antigen stability following inactivation of the virus with formaldehyde. If the factors can be determined which cause the loss in HA antigenicity, an inactivated vaccine will be made, otherwise the inactivated parainfluenza vaccine will be dropped. A workshop will be held on possible hypersensitivity reactions that have occurred in recipients of various inactivated vaccines when they later are infected by that organism.

Rubella: Investigations of the epidemiology, clinical characteristics and viral immunology of rubella, congenital rubella and rubella vaccines have been performed under one longitudinal study. Potential hazards to the fetus by inadvertent vaccination of the mother during pregnancy, efficacy of rubella vaccine-induced immunity and long-term antibody studies are to be continued through other support mechanisms.

Adenoviruses: Crystalline adenovirus type 5 hexon and fiber antigen vaccines were prepared through a collaborative effort of Dr. Julius Kasel, Laboratory of Clinical Investigation, NIAID, and Dr. H. G. Pereira, Mill Hill, England. Both vaccines have been shown to be antigenic and safe in adults, and young teenagers. Dr. Kasel is preparing hexon and fiber antigen vaccines from type 4 adenovirus. The type 4 subunit vaccines will be tested in adults in order to ascertain if they are primary immunogens.

INDIVIDUAL CONTRACTS AND AGREEMENTS TABULATION
INFECTIOUS DISEASE BRANCH

INFLUENZA PROGRAM

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans for FY 1975
AI-02508	University of Virginia	To clinically evaluate live attenuated influenza virus vaccines administered by the nasal route in appropriate adult populations to show infectivity, degree of virulence, and efficacy. Inactivated influenza vaccines are also clinically evaluated. Future objectives are to measure attenuation and infectivity in children and to evaluate vaccine virus spread in family units, and, ultimately, efficacy trials in industrial populations currently under surveillance.	4/1/72	$98,493	Undetermined
AI-[illegible]091	Children's Hospital, D.C.	To evaluate candidate attenuated vaccines of respiratory syncytial and influenza viruses in infants and young children, and to study host factors important for resistance. This facility provides facilities for phase I testing in a pediatric population. The Hong Kong ts-1-[E] attenuated vaccine infected 81% of vaccinated children 3 1/2 to 11 years of age. No local or systemic reaction attributable to the vaccine virus occurred. Each of the virus isolates from the vaccinees retained the ts characteristics.	3/3/71	$101,944 (75% of total contract for $135,[illegible])	Renewal

INFLUENZA PROGRAM (CONT'D)

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans for FY 1975
AI-22507	Vanderbilt Univ.	To maintain a center for clinical evaluation of live virus vaccines in a semi-closed population of infants (>one year old), and young children. A trial of the Hong Kong ts-1-[E] attenuated vaccine was conducted in a classroom. The vaccine was found to be safe, antigenic, and non-transmissible.	5/19/72	$105,430 (75% of total contract for $140,586)	Renewal
AI-22303	University of Rochester	To clinically evaluate viral vaccines as they become available (live and inactivated influenza, live RSV, live and inactivated para-influenza viruses). Twenty volunteers were vaccinated with two live candidate recombinant influenza vaccines (ts-1-[E]-Udorn-72, clones 13 and 16) - ten volunteers per clone. Clone 13 appeared to be underattenuated and clone 16 appeared overattenuated. These findings clearly indicated that the nearly ideal properties of infectivity and attenuation observed with Hong Kong ts-1-[E] attenuated vaccine were polygenic, and would not be solely attributed to the ts lesion.	6/1/72	$ 76,055 (50% of total contract for $152,110)	Renewal

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans for FY 1975
AI-32506	Baylor College of Medicine	To help determine optimal and rational methods for effective vaccination and drug therapy, assess the relative contribution of humoral, secretory and cellular immune mechanisms in host resistance, and develop and then utilize infectious challenge systems as a method for evaluating effects of vaccination and antiviral treatment. The influenza studies during the past year have included development of a challenge system for A/England.	12/20/72	$ 92,400 (40% of total contract for $231,000)	Renewal
AI-[illegible]501	University of Colorado	To conduct laboratory and clinical studies to evaluate efficacy of viral and bacterial vaccines. Evaluation of live attenuated influenza vaccines will be conducted in normal and asthmatic children in the future.	4/4/72	$24,701 (30% of total contract for $82,337)	Renewal
AI-2[illegible]505	University of North Carolina	To conduct clinical evaluation of respiratory vaccines in children and infants. A clinical evaluation of Hong Kong ts-1-[E] was conducted in children. Studies are in progress to determine the occurrence of virus shedding, the antibody response in serum and nasal washings, and peripheral lymphocyte response to the vaccine.	4/1/72	$14,091 (20% of total contract for $70,458)	Renewal

INFLUENZA PROGRAM (CONT'D)

Contract/ Ag. No.	Contractor	Objective & Findings	Date Initiated	Current Annual Level	Plans for FY 1975
AI-32521	Flow Laboratories, Inc.	To prepare and safety test vaccines developed by the LID, NIAID, and contractors of the IDB; and to supply tissue cultures for the LID. Primarily a service contract.	6/23/68	$215,000 (50% of total contract for $430,000)	Renewal
AI-42509	Rutgers University	To evaluate influenza temperature sensitive mutants for phenotype, genetic defects, and infectious potential. Too early for results.	3/11/74	$ 86,008	Renewal
AI-42510	St. Jude Children's Hospital	To isolate and purify various polypeptide antigens of influenza virus. Too early for results.	3/28/74	$ 38,574	Renewal

HEPATITIS PROGRAM

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans for FY 1975
AI-30001	Center for Disease Control	Hepatitis epidemiological survey of two hemodialysis units to determine the incidence of hepatitis B antigen, hepatitis B antibody, and clinical viral hepatitis in the hemodialysis setting; identify factors involved in transmission of hepatitis B or HB_sAg by household contacts of dialysis patients and staff. Too early for definitive results.	7/25/72	$70,000	Undetermined
AI-30522	State University of New York (Buffalo)	To perform an epidemiologic survey of hepatitis B in a population in which viral hepatitis is highly endemic. The study encompasses the descriptive epidemiology of hepatitis B, in both patients and staff, carefully designed to document the incidence and prevalence of HB_sAg infection and illness, and anti-HB_s. Protocols designed, approved, and study underway.	6/18/73	$99,382	Renewal

HEPATITIS PROGRAM (CONT'D)

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans for FY 1975
AI-20002	Veterans Administration	To conduct two double blind studies administering (I.M.) either conventional low titer or high titer anti-HB_s gamma globulin to determine if these preparations lower the incidence of hepatitis B infections in certain "high risk" populations by preventing or modifying the disease. Results are unknown since studies are double blind, but number of subjects at risk in "Needlestick" study has turned out to be less than originally anticipated.	5/1/72	$71,566	Renewal
AI-12197	Tulane University	To provide a facility for studying hepatitis B infections in champanzees. Titration of infectious HBV pools have been completed and pedigreed pools of infectious HBV are being produced.	6/21/71	$24,385	Renewal
AI-30002-01	Albany Medical College	To establish and maintain a breeding colony of chimpanzees. Collaborative effort of NIAID, FDA, and CDC for ensuring chimpanzees for experiments with viruses of hepatitis A and B. Nursery has been established and 12 animals have been provided for experimental use.	6/10/73	$63,000	Renewal

Contract Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans for FY 1975
AI-32504	Meloy Laboratories	Maintain animal holding facility for HBV infected rhesus monkeys. To maintain and periodically sample rhesus monkeys before and after experimental exposure to HB agents. A new capability to maintain chimpanzees and perform experiments with infectious hepatitis A materials has been established. Essentially a service contract.	1/1/72	$200,757	Renewal
AI-32509	Scripps Clinic and Research Foundation	Immunohistochemical assays of hepatitis virus-associated antigens. To develop methods for immunohistochemical assays of intrahepatic HBV-associated antigens and perform pathological studies on primate liver biopsies. Methods have been developed, reagents produced and application is being made in experimental HBV studies in primates.	11/1/72	$79,366	Renewal
AI-40005	Tulane University	To establish and maintain a breeding colony of Saguinus mystax marmosets. Collaborative effort of NIAID, FDA, and CDC for ensuring future availability of small animal model for hepatitis A experiments.	4/22/74	$31,700	Renewal

HEPATITIS PROGRAM (CONT'D)

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans for FY 1975
AI-22522	American Red Cross	To provide a Hepatitis Testing Laboratory to do tests for hepatitis B antigens and antibodies. In addition, the testing laboratory is involved in developing better methods to test for HBV antigens and antibodies. The laboratory provides testing for other ongoing NIAID hepatitis projects, as well as services to other NIH hepatitis research projects.	6/22/72	$457,093 (total est. cost)	Renewal

ANTIVIRAL SUBSTANCES PROGRAM

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans for FY 1975
AI-02125	Medical College of Wisconsin	To develop a reproducible, precise, sensitive, rapid and universally acceptable assay for interferon. Such a test utilizing viral neuraminidase production has been developed and is in its final stages of refinement.	6/1/70	$40,131 "prior year support"	Terminate
AI-02126	Medical College of Pennsylvania	To develop potent and specific antisera to murine and human interferon and to produce crude and partially purified mouse interferon for use by other contractors. Reagent quantities of mouse interferon antisera are now available as is the mouse interferon. The human interferon antisera is under production.	6/1/70	$101,394	Renewal
AI-02169	New York University Medical Center	To develop a more economically feasible method for the production of human interferon which would be acceptable for use in humans. For each year of the contract the investigator has succeeded in increasing the yields several fold; if a 10 fold greater yield can be achieved we will have an economical, useful product. This contract also produces human interferon for purification studies.	5/25/70	$70,096	Renewal

ANTIVIRAL SUBSTANCES PROGRAM (CONT'D)

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
AI-42520	Medical College of Wisconsin	To examine partially purified human interferon in an attempt to stabilize it. As this material is purified for clinical use it loses potency, therefore there is a need for such studies.	6/1974	$60,000	Renew
AI-42514	Medical College of Wisconsin	To lyophilize rabbit interferon and ampule as a research reference reagent; this includes stability studies as well as periodic verification of titer.	4/1/74	$46,749	Renew
AI-32529	University of Pittsburgh	To develop a biological interferon inducer which is effective and nontoxic with clinical potential. Established biological inducers will be compared and the most effective ones chemically characterized. The three phases include: accumulation and purification, comparison and evaluation, and identification of active moiety. This contract will work on Salmonella, Brucella and Xanthomonas organisms.	6/26/73	$81,937	Inc-funded

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
AI-32530	Medical College of Wisconsin	To develop a biological interferon inducer which is effective and non-toxic with clinical potential. Established biological inducers will be compared and the most effective ones chemically characterized. The three phases include: accumulation and purification, comparison and evaluation, and identification of active moiety. This contract will work on E. coli proteins as well as other bacterial and viral products.	6/26/73	$75,001	Inc-funded
AI-32520	Lee Mac	To apply a unique cytogenerator to the production of human interferon. This would be an additional method of reducing the cost of this potential clinical material. The contractor has successfully applied the cytogenerator to the purposes indicated he is currently determining how to improve yields; if high yields can be obtained the contract will be continued; if not, it will be terminated.	6/21/73	$51,960	Undetermined.

ANTIVIRAL SUBSTANCES PROGRAM (CONT'D)

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
AI-42524	University of Utah	To provide a viral disease animal model which closely mimics a disease state in man so as to allow evaluation of promising antivirals as soon as they are identified. This contract is a component of the Cooperative Antiviral Testing Group which has tested several antivirals and continues baseline studies with interferon and its inducers. This particular contract includes herpes virus cytomegalovirus and EMC virus in the mouse.	6/30/74	$80,047	Renewal
AI-02131	New York University	Component of the Cooperative Antiviral Testing Group. This particular contract includes vaccinia, fibroma and herpes viruses as dermatological infections in the mouse and rabbit.	5/25/70	$73,317	Renewal
AI-02132	University of Wisconsin	Component of the Cooperative Antiviral Testing Group. This particular contract includes swine influenza, transmissible gastroenteritis and pseudorabies viruses in the piglet.	6/18/70	$55,767	Renewal

ANTIVIRAL SUBSTANCES PROGRAM (CONT'D)

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans for FY 1975
AI-42523	University of Utah	Component of the Cooperative Antiviral Testing Group. This particular contract is studying the rabies virus model in the mouse.	6/17/74	$73,522	Renewal
AI-02130	Washington State University	Component of the Cooperative Antiviral Testing Group. This contract includes panleukopenia distemper and rhinotracheitis virus in the cat and ferret.	6/1/70	$16,565	To be determined
AI-81266	New York Medical College	Component of the Cooperative Antiviral Testing Group. This contract is studying viral infections (herpes) of the rabbit eye.	6/20/68	$25,000	Renewal
AI-32501	Stanford University	To clinically evaluate the role of exogenous interferon against serious varicella viral infection in immunologically compromised patients. The initial studies to evaluate the possible toxicity of interferon are completed and the double-blind study is well underway.	1/1/73	$87,239	Renewal

ANTIVIRAL SUBSTANCES PROGRAM (CONT'D)

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans for FY 1975
AI-22532	University of Alabama	To clinically evaluate 9-B-D Arabinofuranosyl adenine against herpes encephalitis, disseminated herpes zoster and serious herpes hominis infections particularly in immunologically compromised patients. This is an extensive collaborative program involving approximately 20 clinics. The double-blind study is well underway and progressing well.	6/26/72	$284,189	Renewal

BACTERIAL VACCINE PROGRAM

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
AI-12196	Children's Hospital of Boston	To conduct clinical evaluation of Hemophilus influenzae b polyribose phosphate (PRP) vaccine in infants and to serve as a serologocal reference laboratory for measurement of anti-PRP antibody for IDB meningitis vaccine contractors. Results indicate that antibody response to PRP in infants is low; significance of low antibody level with regard to protection is not yet known. An improved method for performing the radioimmunoassay has been developed which may have an effect on the serological results of the other H. influenzae vaccine contractors when their sera is reassayed.	6/14/71	$115,000	Renewal
AI-22502	University of Connecticut	To carry out clinical evaluation studies of meningococcal group A and C polysaccharide vaccines in infants. No significant local or systemic reactions have been observed with the vaccines. The optimum dose level of group C vaccine for primary immunization was shown to be 100 ug. Booster inoculations were not effective when given at 7 and 12 months following initial dose at 3 months. Data on antibody response in meningococcal group A vaccinees are not complete.	4/18/72	$61,946	Renewal

BACTERIAL VACCINE PROGRAM (CONT'D)

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
AI-22506	University of Southern California	To perform clinical evaluation of Hemophilus influenzae type b, polyribose phosphate (PRP) and meningococcal groups A and C polysaccharide vaccines in infants. Results indicate a poor response by infants under two years of age to PRP and a lower but measurable antibody response to meningococcal group C polysaccharide by children under six months in comparison with older children.	4/4/72	$62,723	Renewal
AI-12502	Rockefeller University	To serve as a reference testing laboratory to measure (using the radioimmunoassay procedure) the serum antibody response of children and infants to immunization with group A and C meningococcal polysaccharide vaccines. Assay of serum specimens from IDB meningitis vaccine contractors is ongoing.	6/28/71	$26,818	Renewal

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
AI-22501	University of Colorado	To perform clinical evaluation of Hemophilus influenzae b polyribose phosphate (PRP) and meningococcal group A and group C polysaccharide vaccines in children. Results thus far indicate that children under two years respond less well than older children to H. influenzae PRP. Likewise, the response of younger children to the meningococcal C vaccine appeared to be less than the response of older ones. Significance of this low level of antibody is to be determined. Preliminary results show that children who had had H. influenzae infections in infancy responded adequately to immunization with H. influenzae PRP, indicating that tolerance may not be induced.	4/4/72	$32,934 (40% of total contract $82,337)	Renewal

BACTERIAL VACCINE PROGRAM (CONT'D)

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
AI-22303	University of Rochester	To conduct clinical studies to evaluate reactivity and antigenicity of candidate bacterial vaccines. Hemophilus influenzae type b polyribose phosphate (PRP) is being evaluated in infants (younger than six months) and polyvalent pneumococcal vaccine in adults over the age of 50. Results indicate that the serum antibody response in children in the 14-18 month age group respond less well to H. influenzae PRP vaccine than those in the 2-4 years of age group. In general, pneumococcal polysaccharides have been demonstrated to be safe and relatively non-reactogenic in elderly patients. The frequency of antibody response was high with all pneumococcal vaccines in all cases.	6/1/72	$45,633 (30% of total contract $152,110)	Renewal

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
AI-32517	University of Pennsylvania	To conduct studies on the epidemiology and immunology of pneumococcal infection and to conduct a field trial to demonstrate efficacy of candidate multivalent pneumococcal polysaccharide vaccines. To serve as a reference serological laboratory to assess antibody response to natural pneumococcal infections and active immunization. Results show that 78% of pneumococcal types isolated from infections belong to the types included in the dodecavalent vaccine currently being tested. Technical assistance is provided by this contractor to the Kaiser field trial (NIAID contract) and to field trials in South Africa (not supported by NIAID contract).	5/31/73	$323,709	Renewal
AI-22513	Kaiser-Permanente	To conduct a field trial to determine the efficacy of a dodecavalent vaccine of pneumococcal capsular polysaccharides in preventing type-specific pneumococcal pneumonia and its impact on pneumococcal pneumonia and total respiratory disease in adults age 45 and older. Approximately 9,000 volunteers have been vaccinated to date. No serious reactions to the vaccine have been observed.	3/1/72	$298,837	Renewal

BACTERIAL VACCINE PROGRAM (CONT'D)

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
AI-22500	University of California	To obtain optimal dosage of octavalent pneumococcal polysaccharide vaccine for maximal antibody response in infants and children. Results of these studies suggest that immunization of infants greater than six months of age with an octavalent vaccine can be accomplished with safety and will result in significant antibody titer increases. Preliminary data indicate that 50% of infants less than six months of age develop low antibody titers to 25 micrograms of the vaccine. Studies on the 50 mcgm and booster doses are in progress.	5/1/72	$60,191	Renewal
AI-81280	Eli Lilly & Company	To prepare monovalent and polyvalent vaccines of pneumococcal capsular polysaccharides and supply them to the IDB contractors for clinical and efficacy studies. Monovalent and polyvalent pneumococcal vaccines have been supplied to University of Pennsylvania, University of California, and Kaiser Permanente contractors for evaluation in clinical evaluation and efficacy trials.	6/28/68	$12,000 "prior year support"	Termination

BACTERIAL VACCINE PROGRAM (CONT'D)

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
AI-00003	Indian Health Service	To conclude by December, 1973, epidemiological studies on the incidence of pneumococcal disease among the Navajo Indian population receiving medical care at the Gallup Public Health Service Hospital, Gallup, New Mexico.	7/1/69	$24,202 (six months)	Termination
AI-12202	University of New Mexico	To complete epidemiological studies on the incidence of pneumococcal disease (pneumoniae, otitis media, and meningitis) in the Navajo Indian population receiving medical care at the Gallup PHS Indian Hospital, New Mexico. Results from this study suggest this subsample, the Navajo Indian reservation, has an exceedingly high incidence of pneumococcal disease (20 cases/1,000 per year).	6/29/71	-0-	Termination

BACTERIAL VACCINE PROGRAM (CONT'D)

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
AI-32519	University of Chicago	To conduct clinical trials to determine antigenicity of streptococcal M protein vaccines in adults. Also, to conduct challenge studies in adults immunized with M protein vaccines. Results indicate intranasal immunization clearly prevented colonization of the pharynx by streptococci used in challenge studies. Preliminary results indicate a similar effect with intranasal immunization with type 12 M protein vaccine.	6/1/73	$115,148	Renewal
AI-32531	Abbott Laboratories	To produce, in lyophilized state, 2.0 gms of crude M protein each of group A streptococci of M-serotypes 1, 6, and 12. This material will be used by several collaborating laboratories to compare methods of purification. The material has been produced and shipped to the participating laboratories.	6/22/73	-0-	Termination

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
(New)	University of Virginia	To test the clinical acceptability, antibody response, and efficacy of a formalin inactivated Mycoplasma pneumoniae vaccine (OSU-1). The clinical tests for reactivity and antibody production will be conducted at the University of Virginia and at the Marine Corps recruit camp at Parris Island, North Carolina. These studies will be followed by an efficacy trial at Parris Island. New contract; no results available.	6/1974	$91,317	Renewal
AI-02065	Cornell University	To serve as a serological testing laboratory for the assay of sera from studies on the clinical acceptability, antibody response, and efficacy of M. pneumoniae vaccines. Safety and potency tests have been performed on the Mycoplasma vaccine to be used in clinical and field trials by IDB contractors. The laboratory is prepared to assay sera from the University of Virginia field trial.	1/18/70	$21,956	Renewal

BACTERIAL VACCINE PROGRAM (CONT'D)

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
AI-32506	Baylor College of Medicine	To evaluate an inactivated Mycoplasma pneumoniae vaccine (OSU-1A) by developing and testing experimental infectious challenge systems for M. pneumoniae following immunization. Results indicate that tissue culture grown M. pneumoniae, when given by small particle aerosal inoculation may produce lower respiratory disease comparable to that seen naturally. The OSU-1A vaccine, while producing local reactions, was relatively non-reactogenic and highly antigenic in terms of frequency of individuals who exhibited an antibody response.	12/20/72	$69,300 (30% of total contract $231,000)	Renewal
AI-70078	Ohio State University	To produce two large lots of inactivated Mycoplasma pneumoniae vaccine for use in the University of Virginia/Parris Island clinical evaluation and efficacy trials. The two lots of vaccine have been prepared and are stored at Flow Laboratories.	12/7/66	-0-	Termination

INDIVIDUAL CONTRACTS AND AGREEMENTS TABULATION
INFECTIOUS DISEASE BRANCH

VIRAL VACCINE PROGRAM

Contract/ Ag.No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
AI-12091	Children's Hospital, D.C.	To evaluate candidate vaccine mutants of respiratory syncytial virus (RSV) and parainfluenza viruses in infants and young children and to study factors that are important in the host's resistance to disease and in the pathogenesis of illness. Infants were immunized with a lowered dose of RSV-ts-1 vaccine. Reduced virulence of the vaccine was not associated with a reduced dose of vaccine.	2/8/71	$33,948 (25% of Total Contract for $135,792)	Renewal
AI-22501	University of Colorado	To evaluate live RSV vaccines and to study parameters of natural infection and pathogenesis of disease. Studies of the RSV-ts-1 mutant vaccine in reduced dosage showed that it spread to contacts and reverted phenotypically to wild type virus.	4/4/72	$24,701 (30% of Total Contract for $82,337)	Renewal
AI-32521	Flow Laboratories Inc.	To prepare and safety-test potential vaccine pools for the LID, NIAID and contractors of the IDB. To provide unique tissue cultures for the LID. To collaborate with Dr. Gerin of the AEC MAN laboratory in the preparation of inactivated parainfluenza virus vaccines.	6/23/73	$215,000 (50% of Total Contract for $430,000)	Renewal

VIRAL VACCINE PROGRAM (Continued)

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
AI-22502	University of North Carolina	To conduct clinical evaluation of respiratory vaccines in children and infants. When a reduced dose of RSV-ts-1 vaccine was administered to four infants, two developed symptoms that were attributable to the vaccine. Intensive studies on the epidemiology of RSV are in progress.	4/1/72	$56,367 (80% of Total Contract for $70,458)	Renewal
AI-22503	University of Rochester	To clinically evaluate live RSV and live and inactivated parainfluenza virus vaccines as they become available. Data were obtained in three children that the RSV-ts-1 vaccine was insufficiently attenuated for use in infants.	6/1/72	$30,422 (20% of Total Contract for $152,110)	Renewal
AI-22504	Rutgers University	To develop attenuated mutants of RSV and parainfluenza virus for use as potential vaccines. Forty RSV-ts clones are being tested for stability of the ts property. Mutagenesis of the parainfluenza viruses has been initiated.	6/1/72	$108,099	Renewal
AI-22507	Vanderbilt University	To conduct clinical evaluations of respiratory virus vaccines in infants and children. Nine of 25 infants immunized with RSV-ts-1 were infected. Vaccine virus was not transmitted to contact controls.	5/19/72	$35,146 (25% of Total Contract for $140,586)	Renewal

Contract/ Ag. No.	Contractor	Objectives and Findings	Date Initiated	Current Annual Level	Plans For FY 1975
AI-92222	Roosevelt Hospital	To conduct longitudinal studies of rubella virus vaccine efficacy and safety, to study natural history of rubella and congenital rubella, and to develop improved techniques for characterizing the immunology of rubella infection. Slight declines have occurred in the geometric mean titer of vaccinees 5 years after immunization. Rubella vaccine does not appear to be a cause of progressive joint disease. Cell-mediated immunity appears to play a role in protection against natural rubella virus challenge.	6/30/69	$130,457 "Prior year Support"	Terminate
AI-32506	Baylor College of Medicine	To develop and evaluate crystalline subunit adenovirus vaccines. Adenovirus type 5 hexon and fiber vaccines have been shown to immunogenic and essentially non-reactogenic in adults and children. The fiber vaccine appears more immunogenic than the hexon vaccine.	1/1/74	$69,300 (30% of Total Contract for $231,000)	Renewal
AI-40003	St. Louis University	To analyze human cancers for single copies of gene sequences specific for human adenovirus types 2, 7, and 12. No results are available yet.	1/1/74	$10,000 (NIAID's portion of Total FDA Contract for $71,850)	Renewal

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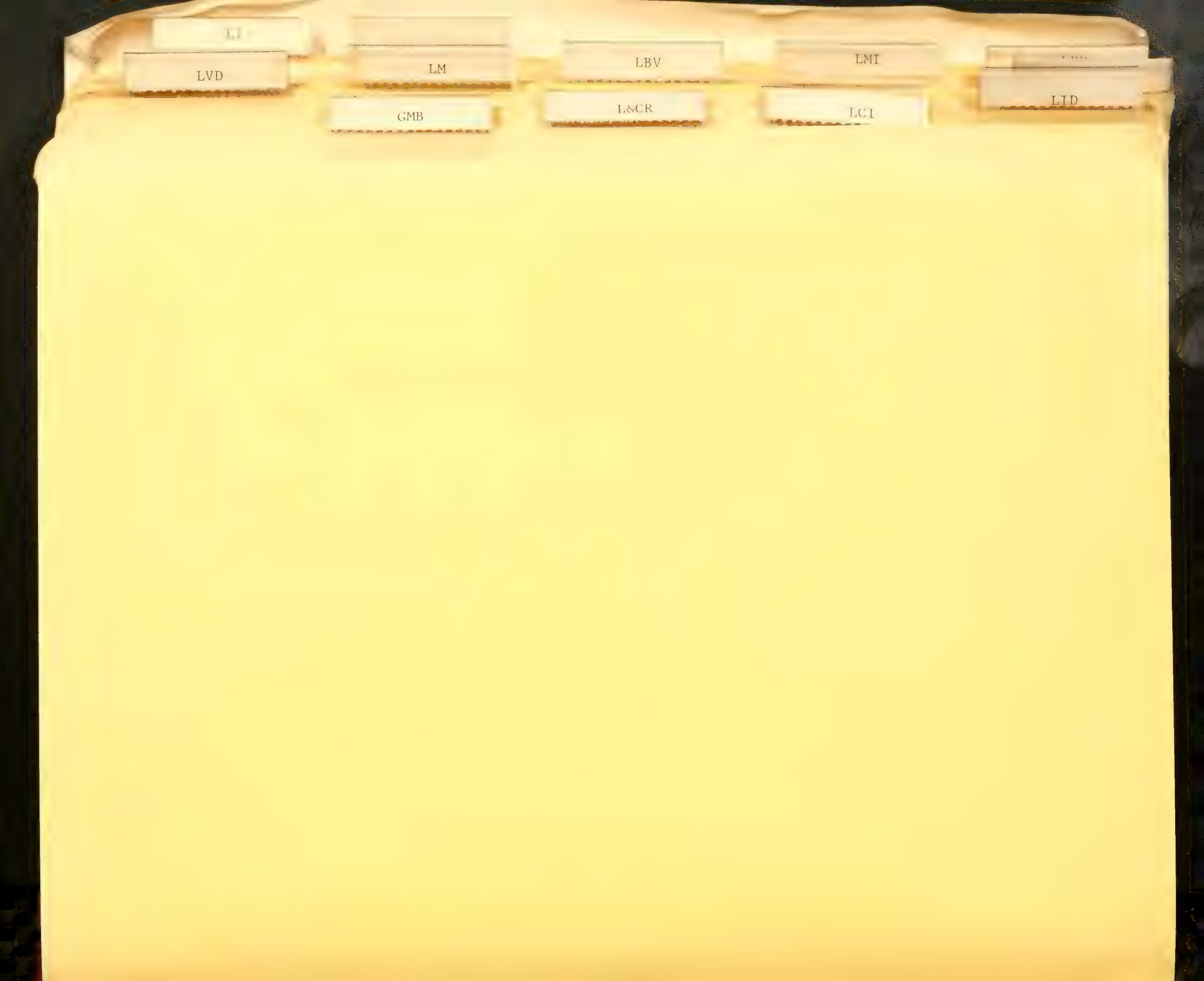
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GEOGRAPHIC MEDICINE BRANCH

The Geographic Medicine Branch was created on 1 July 1968 to manage several biomedical programs transferred to the National Institute of Allergy and Infectious Diseases (NIAID) from the Office of International Research. The Branch is currently part of Collaborative Research Programs, NIAID, and consists of two principal organizational elements: The U.S.-Japan Cooperative Medical Science Program Section and the International Centers for Medical Research Section (ICMR).

Grants and contracts are used in support of U.S.-Japan program, but grants are the only support instrument for the International Centers. For a detailed history of these programs and their operating methods, the reader is referred to the NIAID Annual Report for FY73 page 1 through 14.

The Joint Committee and the Program Panels meet annually, alternating between the United States and Japan. This year all of the meetings will be held in Japan. At the Joint Committee meeting, August 8 and 9, 1974, the Program reviews of the Malnutrition and Viral Diseases Panels will be completed, and the Committee will make definitive plans to publish a second Five Year Report.

I. The United States - Japan Cooperative Medical Science Program

Highlights of the Past Year

CHOLERA

The Cholera Program has made advances in producing a toxoid for use in a planned field trial, in continuing studies on oral immunization, and in identifying a chemical which shows promise in controlling fluid loss.

Over 100,000 doses of purified cholera toxoid await final clearances on standard safety and toxicity tests prior to testing in human volunteers. In the production of this toxoid, the manufacturer instituted a new step in the purification procedure which reduced the somatic antigen level to 0.1 that of the previous accepted standard. This is by far the purest toxoid we have available and should answer the all important question of the effect of anti-toxic immunity in protecting against cholera in an endemic area. A preliminary test of safety and immunogenicity in the endemic area of Bangladesh (after appropriate studies in U.S. volunteers) demonstrated that this toxoid produced high levels of circulating antitoxin and very minimal local reactions in residents of the endemic area. The toxoid appears to be stable in the lyophilized form for over one year at 37 and 45°C. Stability in the liquid form is about one month at these temperatures.

The advent of an oral cholera vaccine may offer a new approach to cholera immunization. It is believed that the fluid loss caused by cholera toxin is due to the attachment of the toxin to the cells of the intestine. Animal studies indicate that locally produced antibodies in the intestine may interfere with this attachment of the cholera toxin to the intestinal cell wall.

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Further studies are needed to determine the duration of this local immunity.

In screening chemicals which might inhibit the fluid loss caused by cholera toxin, zirconyl oxycholoride has produced some exciting results. Preliminary data in dogs indicate that fluid loss is greatly reduced after oral administration of zirconyl oxychloride and, in some instances, the fluid loss is stopped in 30 minutes.

Similarities between the heat-labile enterotoxin of Escherichia coli and V. cholerae enterotoxin continue to be demonstrated and their immunological relationship has been more firmly established by cross-neutralization tests. It has been shown that E. coli enterotoxin causes an increase in permeability of the small blood vessels of the skin, similar to, but not identical with that caused by cholera enterotoxin. This can now be made the basis of a method of screening strains for enterotoxinogenicity and for titrating antitoxin. The crude E. coli filtrates thus far prepared contain far less biologic activity either in gut or in skin than do cholera filtrates.

Progress has been made in purification of E. coli enterotoxin, although this has proven to be much more difficult than in the case of cholera toxin. Affinity chromatography using cholera antitoxin, and gel filtration have both met with some success. Skin reactivity is separable into a heat-labile factor which increases vascular permeability and into a heat-stable factor which causes "blanching." These findings may help explain the qualitative difference between the typical skin lesions produced by E. coli and cholera toxins.

Because of these recent rapid advances in our knowledge of E. coli enterotoxins and in the development of simple screening methods for the detection of enterotoxinogenicity, Program has decided that a study should be carried out to determine the magnitude of the disease problems caused by this group of organisms. The aim is to develop a clearer picture within the next few years of the overall impact which this group of agents exerts on human morbidity and mortality. Any future consideration of control measures, especially of immunizing agents, must be founded upon a sound understanding of the natural history of this group of diarrheas.

ENVIRONMENTAL MUTAGENESIS & CARCINOGENESIS

During the past year, the Environmental Panel continues to emphasize the importance of monitoring environmental chemicals for potential mutagenic and carcinogenic effects. At the Joint U.S.-Japan Environmental Conference this year, the potential hazard of the Japanese food additive, AF-2, was discussed and plans to study this chemical in this country were formulated. AF-2, a nitrofuran compound, has been used as a food additive in Japan since 1965, and has recently been found to be mutagenic in yeast, salmonella, and neurospora test systems.

An attempt to stimulate research in the development of methods for detecting mutagenic and carcinogenic activity of chemicals was initiated by alerting the scientific community to program needs. As a result of this, research

was recently funded to develop better assay systems with drosophila.

A workshop on "Long-term Toxicity of Antischistosomal Drugs," was jointly sponsored with the Parasitic Diseases Panel. The major concern of the workshop was the ability of drugs to cause genetic activity in human populations. Two productive days of discussions among the 35 participants resulted in recommendations for screening potential mutagenic and carcinogenic agents that might expose large populations.

LEPROSY

During the past year the Leprosy Program has placed greater emphasis on the immunology of leprosy. Preliminary studies of the peripheral blood B:T lymphocyte ratio among patients with leprosy are in progress. The evidence strongly suggests that patients with lepromatous leprosy may have profound disturbance of the blood B:T cell ratio. For example, these patients may show absolute decreases in the percentage of blood T-cells in association with marked increases in absolute percentages of B-cells. The question of what happens to blood T-lymphocytes in leprosy is believed to be of fundamental importance in view of the energy displayed by these patients. Such questions as to whether T-cells are destroyed, or trapped, and whether return to normal blood ratios of B:T cells occurs under therapy are all basic to the understanding of the immunological features of the disease.

Through a contract with the University of Hawaii, additional epidemiological data has been gathered regarding the effects of a three-year (1968-1970) mass chemoprophylactic treatment for leprosy with acedapsone (DADDS) in the isolated Pingelapese population. The results of the third annual surveillance of this population are as follows: a significantly lower incidence of leprosy among those individuals who received a full 3 year course of DADDS; and no cases have yet appeared among 300 or more children born since 1968, and who did not receive DADDS prophylactically.

It should be noted these children are members of families whose parents were participants in the mass chemoprophylatic treatment program. This preliminary evidence suggests the effectiveness of prophylactic DADDS continues after you stop administering the drug and this effect is truly preventive, and not merely suppressive. It is anticipated that information obtained from this study will significantly facilitate the establishment of control programs in leprosy endemic areas with limited medical and public health resources. Periodic surveillance of this population group will continue through 1980.

A variety of pharmacologic studies have been carried out during the past year using the mouse foot pad technique. These include presence of viable organisms from patients on chemotherapy and sensitivity levels of candidate antileprotic drugs.

The minimum effective dose (MED) of dapsone for M. leprae in thymectomized-irradiated mice was found to be identical with that of the normal immunologically intact animal. The protein-binding of dapsone was studied, as well as its disposition in a number of animal species. These studies have

pointed to an important route of elimination of the drug from the body that yields metabolites in the urine not detectable by the usual analytical techniques. Finally, hydnocarpic acid, a unique fatty acid that is a major component of chaulmoogra oil, was found to possess antimicrobial activity against a large number of cultivable mycobacterial species and strains, and to act by an unusual mechanism that involves biotin.

In the field of experimental pathogenesis, multiplication of M. leprae but not of M. marinum was inhibited by prior infection of mice with the protozoans Toxoplasma gondii or Besnoitia jellisoni. Multiplication of both mycobacterial organisms was also inhibited in mice previously infected with either, thus demonstrating both homologous and heterologous immunity.

Evidence accumulates from chemotherapeutic trials conducted at the Cebu, Philippines, Division of Leonard Wood Memorial, that Rifampin is the drug of choice for its rapid bactericidal activity and safe record for adverse reactions.

The possibility of using transfer factor for immunotherapy of lepromatous leprosy continues to attract wide interest. Depending on the outcome of independent pilot studies (not sponsored by NIAID) conducted at the USPHS Hospital, Carville, La., and elsewhere, the U.S. Leprosy Program has definitive plans under consideration for a well-controlled clinical study to determine the advantage of this type of immunotherapy.

MALNUTRITION

Among the various highlights of the past year was the contract support for a scientific conference January 1974, at Guatemala City entitled, "The Latent Effects of Malnutrition and Infection During Pregnancy as Determinant of Growth and Development of the Child." Participants focused attention on malnutrition during pregnancy with discussions of placental function with emphasis on dietary intake, types of dietary supplements and the optimal time for supplementation of the mother so that effective transplacental transfer mechanism's could effect the optimal response. In addition, the biochemical, metabolic and physiologic consequences of cell damage by microorganisms were discussed. The difficulties and methods of diagnosing intrauterine infection were also stressed.

Consideration was also given to the significance of asymptomatic bacterial infections during pregnancy which influence low birth weight. Attention was also given to the long lasting implications of low birth weight in respect to survival and growth of the child as well to psychomotor development. Final edited material from this conference will be prepared for publication and dessiminated to the scientific community.

During the past year, the "Food Composition Table For Use in East Asia" was completed and published. This comprehensive reference provides valuable information concerning numerous studies on the subject and will be extremely useful in the evaluation of Asian dietary consumption and in the assessment of the nutritional status of the population in the various countries of East Asia.

Investigators at Ramathibodi Hospital, Bangkok, Thailand, have demonstrated bladder stone disease in children is a major health problem. In Thailand, the prevalence rate is 12 per 1000 population in rural areas of the North and Northeast regions.

Evidence accumulated in Thailand over the past several years supports the concept of a nutritional etiology of bladder stone disease. It appears that phosphate deficiency, high oxalate intake, protein malnutrition and possibly other factors are important in stone formation. Infants and children in endemic stone areas show very low levels of phosphate excretion, somewhat high levels of urinary oxalate and also oxalcrystalluria. Administration of orthophosphate salts to these subjects dramatically reduced the occurrence of oxalcrystalluria.

In a collaborative study conducted at Chiang Mai, Thailand, the importance of infection in the morbidity and mortality associated with protein-calorie malnutrition has been well established. In 200 children, 1 to 4 years of age, with second and third degree malnutrition, 69% were discovered to have potentially life threatening infections which included diarrheas of bacterial and viral origin, pneumonia, otitis media, gastrointestinal infections, and skin infections. Thirty percent had sepsis, mostly of gram negative origin associated with detectable amounts of endotoxin in the plasma. It was discovered, furthermore, that children with severe protein-calorie malnutrition have an enhanced incidence of hepatitis-associated antigen (HAA) which suggested a defect in cell mediated immunity. Skin testing with nitrofluorobenzene, monilia and streptokinase-antigens also revealed a defect in cell-mediated immunity in these children which was corrected by improved nutrition as reported recently in Lancet. It was also found that blast cell transformation of lymphocytes stimulated with phytohemogglutinin and thymidine uptake was also reduced as additional evidence of alterations in the T-cell system in these children.

PARASITIC DISEASES

Research in the Parasitic Diseases Program is confined to two parasitic infections of very great importance in Asian countries - schistosomiasis and filariasis

Schistosomiasis

Work toward the objective of establishing a cell line of the snail host of *S. mansoni* has progressed well this year. Culture techniques have been refined and mitotic figures have been observed as late as 50 days after the establishment of the culture. Larval stages of *S. mansoni* are being cultivated and their metabolism monitored.

Studies on the mechanism of immune response have been expanded during the past year. It was shown that T-lymphocyte depletion prevented the development of anti-schistosomal egg antigen (SEA) cell-mediated immune responses and anti-SEA reaginic antibody. Other studies have been initiated to help elucidate the function of the eosinophil in the destruction and elimination

of schistosomal eggs from the body.

Suppression of schistosome granuloma formation was brought about experimentally by cholera toxin, by diabetes and by toxoplasmosis.

A series of studies on the effects of a nitrovinylfuran compound on Schistosoma japonicum in chimpanzees showed that treatment with this compound had a pronounced effect on diminishing the extent of colonic leisons and on lessening the degree of schistosomal nephropathy.

Filariasis

Work on experimental models of human filariasis continue to corroborate the finding that the gerbil is the most useful rodent host for studies with the lymphatic-dwelling filarial worms. Basic data have been obtained on the factors underlying the susceptibility of male gerbils to infection. For the first time, W. bancrofti, the human filarial parasite, has been introduced into primates with production of microfilariae. If this work can be extended, the door will be opened to a wide range of studies on this important parasite.

Biochemical studies have included comparison of the metabolism of L. carinii, D. wifei, and B. pahangi. D. wifei and B. pahangi survive anaerobically for extended periods of time, whereas L. carinii loses motility rapidly in the absence of air. This may explain the ineffectiveness of cyanines in human infection. It has also been found that stibophen inhibits phosphofructokinase of all three species of filarids, but not that of the host. Incubation of microfilariae in immune serum with radioactive diethylcarbamazine resulted in a three-fold increase in drug uptake.

Immunization of dogs with a single subcutaneous dose of gamma-irradiated infective third-stage larvae of Brugia pahangi resulted in a substantial degree of protective immunity against homologous challenge infections.

Ninety carefully selected compounds were tested for chemotherapeutic and/or chemoprophylactic activity against filarial infection. The Brugia pahangi gerbil system seems promising as a small animal model for evaluating filariasis chemotherapy.

TUBERCULOSIS

The research sponsored this year by the Tuberculosis Program was again focused on the immunological aspects of tuberculosis. Through several service contracts, highly characterized and standardized mycobacterial materials are made available to qualified scientists in tuberculosis and related fields. An unheated mycobacterial culture filtrate preparation was fractionated by gradient acrylamide gel electrophoresis (GAGE) and by ion exchange and molecular exclusion chromatography. The isolated antigenic fractions obtained by these two methods will now be studied by in vivo and in vitro test systems to determine the immunologically important and biologically reactive fractions.

Because of the great demand for the immunoelectrophoretic reference system for antigems of Mycobacterium tuberculosis, the original supply of antigen and antiserum were depleted. A new supply was produced this year and the relationship of the new materials to the original system was established. The reference kits are again available for distribution.

Research was initiated to systematically study mycobacterial antigens obtained using various cell disruption techniques. Virulent human tubercle bacilli were disrupted by several sonication and pressure cell regimens and the protoplasmic constituents of these preparations were compared. The differences in antigens that can be isolated from the various preparations can now be studied.

The monkey model for studying aerosol BCG vaccination with aerosol challenge was investigated further. It was shown that the protection of aerosol BCG vaccination is dose dependent; i.e., 10^5 organisms gave a greater degree of protection than either 10^3 or 10^4 organisms. There was some concern that repeated vaccination via the aerosol route might elicit a type of hypersensitivity reaction in the host. Therefore, guinea pigs, an excellent model for exploring such a response, were sensitized and vaccinated with aerosolized BCG organisms. No adverse effects were observed.

Advances in the basic immunology of the mycobacterioses were seen. Improved methods of studying the blastogenic response of human leukocytes has made it possible to determine qualitatively and quantitatively the immunologic status of individuals. The role in cellular immunity of macrophages, B and T lymphocytes, soluble mediators, surface antigens, and a battery of enzymes was further clarified by continuing research.

VIRAL DISEASES

Research in the Viral Diseases Program is limited to studies on rabies and on arboviruses with emphasis on dengue and the pathogenesis of hemorrhagic fever.

Rabies

Work has continued on the isolation and characterization of the protective antigens of rabies virus. Guanidine extraction has been shown to result in products of low immunogenicity. In contrast, Triton, a non-ionic detergent, has been found to yield a high titer product. The results demonstrated that dissociation of virus particles did not inactivate the protective antigen and that the solubilized antigen was an effective immunogen.

Also being developed are improved methods for the inactivation of rabies virus without significant loss of immunogenicity. Twelve consecutive 10 liter lots of rabies virus have now been inactivated by betapropiolactone at a concentration of 0.025%. No residual live virus has been found in any of these lots as measured by the sensitive rabies virus amplification procedure.

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An *in vitro* test for the evaluation of the potency of rabies vaccines by the stimulation of sensitized lymphocytes was elaborated. Several vaccine preparations of tissue culture origin were tested and compared with several preparations of the standard NIH vaccine. Results indicate that a reasonable degree of correlation can be obtained between the potency measured in experimental animals and that measured by *in vitro* procedures.

Dengue

Studies are being directed at determining whether susceptibility of mosquitoes to dengue viruses is genetically controlled. The infection in the mosquito is also being studied quantitatively. Of 11 different geographical strains of *Aedes albopictus* which have been screened for susceptibility to dengue viruses, 2 consistently showed lower infection rates and 1 strain showed a higher rate than the others.

In a study of the ecology of dengue viruses in Malaysia, an area of undisturbed primary rain forest was chosen as the site of investigation. Serological conversions for dengue were detected in three leaf monkeys in the high canopy at about 75 feet above ground. The results have established that enzootic dengue occurs in the high canopy of the study area. A search continues for the proven forest vector.

Attempts are being made to develop temperature-sensitive mutants of dengue viruses as potential seed material for live attenuated vaccines for human use. Of several hundred clones (after ten passages) analyzed, 8 were found to be temperature sensitive.

Pathological studies of dengue infections have involved the development of quantitative techniques for the detection of viremia, techniques for purification of type-specific viral antigens and antibodies for specificity studies, and the physico-chemical characterization of viral antibody complexes.

Research Conferences and Workshops:

In addition to program elements involving research grants and contracts, the U.S.-Japan Cooperative Medical Science Program includes a provision for regular scientific conferences and workshops. These include joint meetings with Japanese counterpart scientists as well as special conferences for American investigators working in the scientific areas of special interest to a given Panel. These workshops and conferences serve to stimulate interest and enthusiasm which leads to the development of new scientific approaches to the seven disease categories within the Program, and they also facilitate the timely exchange of research data, methods and protocols. The Joint U.S. and Japanese Panels meet annually, alternating between locations in the United States and Japan. A notation of conferences and workshops sponsored during FY 1974 follows:

Meeting Title and Location	Sponsoring Panel
July 23-25, 1973 Joint Malnutrition Conference Bethesda, Maryland	Malnutrition
July 23-25, 1973 Joint Viral Diseases Conference Bethesda, Maryland	Viral
July 26-27, 1973 Joint Committee Meeting Bethesda, Maryland	
July 30-31 & Aug. 1, 1973 Joint Leprosy Conference San Francisco, California	Leprosy
August 6-8, 1973 Joint Parasitic Diseases Conference Monterey, California	Parasitic Diseases
September 19-20, 1973 Rabies Workshop Bethesda, Maryland	Viral
October 1-3, 1973 Joint Cholera Conference Grand Canyon, Arizona	Cholera
November 12-14, 1973 Joint Tuberculosis Conference New Orleans, Louisiana	Tuberculosis
November 19-21, 1973 Joint Environmental Conference Charleston, South Carolina	Environmental
January 10-12, 1974 Malnutrition Workshop Guatemala City, Guatemala	Malnutrition
January 24-25, 1974 Joint Subcommittee Meeting Honolulu, Hawaii	
May 1-3, 1974 Parasitic & Environmental Panels Workshop Bethesda, Maryland	Parasitic Diseases & Environmental

II. The International Centers for Medical Research (ICMR) Program

Program Description:

In response to the expressed desire of the Congress (P.L. 86-610), a program for the International Centers for Medical Research was established in 1960 by the National Institutes of Health "to advance the status of the health sciences in the United States and thereby the health of the American people" by expanding collaborative research between U.S. universities and selected foreign institutions and investigators.

The four grants funded under this Program are awarded for five years with continued support contingent upon program review at the end of each year of operation. One of the most important features of this program is that of long-term support, as contrasted with the transiency which has characterized a number of other international projects. This is a necessary ingredient for the full development of university careers in science which will effectively utilize the international medical experience of individual scientists. A major advantage of this program is that it provides opportunities for established and highly competent investigators to exploit valuable research opportunities abroad without losing their university affiliations and faculty appointments. The long-term support of the ICMR's does not represent a form of foreign aid. Rather the primary objective is a collaborative effort in biomedical research of specific relevance to the health of the American people and to the U.S. scientific community. The four current ICMR grants were renewed for an additional five years beginning in FY 1970, and are presently undergoing scientific evaluation for further support.

Purpose and Scope:

The ICMR program has as its principal objective the provision of high quality research and scientific opportunities for Americans in the broad fields of geographic medicine, and in response to the special opportunities existing within each ICMR framework. The length of the research period overseas is a discretionary concern of the ICMR program director, and is obviously determined by both the interests of the prospective investigator and the specific opportunities available within the given ICMR unit. Consistently, the emphasis is upon the quality rather than the quantity or duration of the research programs.

The aggregate ICMR units are serving increasingly as a national resource for utilization by senior as well as less experienced scientists to create a comparatively modest pool of investigators with a sustained career interest in international or geographic biomedical research. In this regard, the ICMR program directors are encouraged to establish a selective, interdisciplinary scientific program. Their activities encourage and accommodate a number of parent university departments other than the department which primarily sponsors the ICMR operation. Particular advantage is taken of special strengths within the overall parent university, including opportunities in health-related fields outside of the classical confines of tropical medicine.

During FY 1971, the NIH initiated a program element within the overall ICMR framework. Designated as the ICMR Research Associate Program (IRAP), this centrally coordinated activity is specially designed to recruit investigators on a national basis and specifically from universities that do not have an ICMR.

As far as is possible, the ICMR core grant is used for research support at the off-shore ICMR site rather than at the parent, domestic university. In this regard it is accepted that some preparation preliminary to an overseas assignment is frequently necessary or desirable, and as many items of equipment and supplies as feasible are purchased in the United States.

Program:

The four institutions participating in the ICMR program at present, and their respective areas of research interests are as follows:

Johns Hopkins University (Baltimore, Maryland)

Program Director: Dr. Frederik B. Bang

During the past year, Johns Hopkins University has transferred its operations from Calcutta to Dacca, Bangladesh to take advantage of the facilities at the Cholera Research Laboratories and to initiate a series of studies on Bangladesh disease problems which had been previously studied by the Hopkins group in West Bengal and India. Program emphasis will continue to be directed toward the epidemiology and management of human diseases of large population and the ecology and behavior of animals which live in comparatively close association with man. On-going research includes cholera and cholera-like disease. Epidemiological studies of respiratory diseases and infectious hepatitis are also in progress. Other research includes ecology of the pariah dog, the dung beetle and various rodents. Nutritional research involves the effect of malnutrition on immune cells, the effect of vitamin A and psychological correlates of malnutrition. A program of research on urban filariasis has just been initiated. It will emphasize quantitative aspects of transmission, model building and immune response.

University of Maryland (Baltimore, Maryland)

Program Director: Dr. David F. Clyde

The University of Maryland with the participation of its School of Medicine, and collaborating with the Institute of Hygiene, Lahore has undertaken a varied research program in West Pakistan. Comprehensive studies of arthropod-borne diseases have been undertaken. During FY 71, a field unit in Salvador (Bahia), Brazil was established at the Federal University of Bahia to study insect vector pathology as a tool for biologic control of malaria. Additional studies in the area of schistosomiasis and vector genetics have also been carried out. Plans are currently underway to phase out the unit in Brazil, although studies on mosquito genetics will continue for another two years.

More recent research in Pakistan includes studies of mosquito-borne viruses in man. In addition, a detailed investigation of several different clinical entities, e.g. bleeding disorders in humans, has been recently completed. Research on immunity in tuberculosis and on the relationship between cirrhosis, hepatitis-associated antigen, and viral hepatitis has also been completed.

Mosquito genetic investigations of a comparatively unstudied vector of Japanese encephalitis virus have been of unusually high scientific merit and promise. Knowledge about the genetics of this mosquito, Culex tritaeniorhnychus, now approaches the abundance and high quality of information previously known about two other mosquito vectors of human disease. This new information has been sought to establish a scientific basis for the ultimate biologic control of human Japanese encephalitis. A new "compound chromosome" technique has been developed and is under consideration for early field application on a limited scale.

University of California (San Francisco, California)

Program Director: Dr. J. Ralph Audy

The University of California with the participation of the Hooper Foundation, the School of Public Health, and the Medical School, is collaborating with the Institute for Medical Research, Kuala Lumpur, Malaysia, and the University of Malaya. This International Center continues to place strong emphasis on infectious diseases transmitted from animals to man. Because of the long standing research interest and experience in studying the fundamental problems of human ecology at the University of California, a collaborative program has been developed embracing medical zoology, microbiology and parasitology. The scope of this collaborative endeavor has now been expanded to include related socio-cultural research and medical genetics.

Long-term research projects in Malaysia and California are based on three general laboratory programs: the Arbovirus Research Unit, the Parasitology Laboratory, and the Medical Anthropology and Rural Health Research project.

The arbovirus research is focused on the epidemiology and ecology of dengue virus and the relationship of this virus to mosquito-borne hemorrhagic fever. A jungle reservoir (monkey) has recently been demonstrated for dengue. The parasitological research is based on studies of trematode ecology and interactions of various parasites in the snail host and the ecology of Malayan pentastomes or tongue worms. The snail trematode antagonism studies are directed toward the biological control of schistosomiasis, and this ICMR represents a leading world resource for this important field research. Investigators from this ICMR have also studied the histopathology of host reaction to acarine parasites, the relationship of monkeys to human malarial infection, and jungle cycles of scrub thyhus, Q fever, and tick typhus.

Abundance, distribution, life cycles and ectoparasites of rodents closely associated with man have also been studied. Work on the possible role of the Indian fruit bat as a reservoir of human viral infections has been completed. Other field studies include the investigation of bacteria and viruses pathogenic for man in monkey populations. Socio-cultural studies concerned with Malayan attitudes toward health and nutrition and the influence of native and western practitioners of medicine upon these attitudes have been undertaken by a number of variously trained, medically oriented social scientists.

Tulane University (New Orleans, Louisiana)

Program Director: Dr. Paul C. Beaver

Tulane University, with the participation of the Department of Tropical Medicine and Public Health, other departments of the Medical School, and the Department of Anthropology and Sociology, is collaborating with the Universidad del Valle, Cali, Colombia. The research work is in two general areas: causes, prevalence and distribution of human diseases with human relevance, and physical environments which determine ill or well-being.

Parasitic diseases research has continued to focus principally around the following diseases: trypanosomiasis, malaria, filariasis, and amebiasis. The local epidemiology of Venezuelan Equine Encephalitis has been studied and documented following the recent epidemic. The Virus Group also has a continuing interest in rabies. A comprehensive clinical evaluation of bacterial meningitis treatment regimens is in process. Finally, the multi-disciplinary study of frustrated suicides has recently been continued and refined. New projects are concerned with maternal and child health, family planning, and include health services research.

INDIVIDUAL CONTRACT TABULATION
Geographic Medicine Branch
Contracts and Agreements
Cholera Program

Contract Number	Contractor	Objectives & Findings	Date Initiated	Current Annual Funding	Plans for F.Y. 1975
NO1-AI-73-2502	Jefferson Medical College	Operation and Maintenance of a Cholera Vibrio Reference Laboratory. Makes and distributes cholera typing sera. Essentially a service contract.	9/5/72	$30,857	Renewal
NO1-AI-70-2751	Johns Hopkins University	Evaluation of Purified Cholera Toxins and Toxoids as Immunizing Agents Against Cholera. Essentially a service contract.	12/20/69	$76,000	Renewal at reduced level
NO1-AI-70-2091	Research Foundation State University of New York	Operation of a Laboratory for Evaluation and Measurement of Cholera Toxins, Antitoxins and Toxoids. Essentially a service contract.	4/16/70	$94,320	Renewal
NO1-AI-70-2101	The University of Texas Medical Branch at Galveston	Study of Cholera Toxoids in Human Volunteers. Essentially a service contract.	5/27/70	$40,000	Renewal
NO1-AI-73-2514	Wyeth Laboratories, Inc.	Production and Purification of Cholera Toxin and Vaccine (Toxoid) Production. Toxoid for field trial is now in final stages of production.	3/24/70	0	Termination

INDIVIDUAL CONTRACT TABULATION
GEOGRAPHIC MEDICINE BRANCH
Contracts and Agreements
Leprosy Program

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans for FY 75
NO1 AI 72-2075	University of Hawaii	Collection and analysis of data on Leprosy in the Pingelapese population. This study will not be completed until 1980. It is anticipated the prophylactic effect of DADDS therapy and the required threshold dose of this drug to interrupt the transmission of leprosy will be ascertained.	1/6/72	$ 7,810	Renewal
NO1 AI 70-2283	Stanford Research Institute	Study the metabolism of dapsone (DDS) in leprosy patients receiving this drug or DADDS for therapy. The half time of disappearance of DDS averaged 18 hrs. and does not appear related to phenotype.	6/25/70	64,296	Renewal
NO1 AI 73-2528	Leonard Wood Memorial	Conduct clinical trials of candidate chemotherapeutic compounds in leprosy patients in Cebu, Philippians. Long term response to various drug regimens- rifampin plus dapsone and rifampin plus DADDS are evaluated in lepromatous patients. Rifampin appears to be bactericidal and reduces the BI (bacterial index) more rapidly than DDS or DADDS.	3/18/69	150,000	Terminated

INDIVIDUAL CONTRACT TABULATION
GEOGRAPHIC MEDICINE BRANCH
Contracts and Agreements
Leprosy Program

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual level	Plans for FY 75
2-YO1-AI 90905-04	USPHS Hospital/ San Francisco	To perform dupiicate mouse footpad inoculation of skin specimens submitted from leprosy patients on study at Cebu, P.I.; determine dapsone susceptability of M. leprae isolates. More than 1200 mice were inoculated, 220 mouse footpad sections prepared and studied. Duplication of the results of the Cebu investigators has been excellent during FY 74.	7/1/73	$30,000	Renewal
2-YO1-AI 10004-02	Center for Disease Control	Continue to investigate the activity of Dapsone and other anti-leprotic drugs using a pharmacokinetic method in the mouse footpad model. Drug combinations are currently being evaluated for DDS-resistant infections. This includes B663 and rifampin: Cephaloglycin appears to be active by injection whereas cephalexin appears inactive.	7/1/73	81,568	Renewal

INDIVIDUAL CONTRACT TABULATION
GEOGRAPHIC MEDICINE BRANCH
Contracts and Agreements
Malnutrition Program

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual level	Plans for FY 75
NO1 HD 72-2737	Pan American Health Organization	Analysis of data from an ecological study of infection of malnutrition and growth of children in a Guatemalan Indian Village. Dr. Mata is now completing a monograph detailing the characteristics of the village, methods of investigations and the extensive conclusions derived from detailed analysis of the data. It is anticipated the monograph will be completed by summer, 1975.	4/1/72	$64,450	Renewal
NO1 HD 74-2816	St. Joseph Hospital	Instrumentation Design for Electrophysiological Studies. The portable electronic aquisition system is now in the operational prototype stage, capable of producing programmed pattern stimili and recording two channels of electrophysiological data along with three event monitors.	1/1/74	90,000	Renewal

INDIVIDUAL CONTRACT TABULATION
GEOGRAPHIC MEDICINE BRANCH
Contracts and Agreements
Malnutrition Program

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual level	Plans for FY 75
NO1 HD 73-2703	Harvard University	Studies on Malnutrition and Mental Development. This contract supported exploration of the possible relationship between nutrition and mental development in children living in an urban ghetto environment. The study has shown that the supplementation system works in terms of improved growth rate of the children.	6/21/68	$35,550	Terminated
74-C-374	National Academy of Sciences	Arrange and conduct conference on "The Effects of Malnutrition and Infection During Pregnancy as Determinants of Growth and Development of the Child". This conference was held January, 1974 at the Institute of Nutrition for Central America and Panama in Guatemala City.	10/23/73	19,113	Complete

INDIVIDUAL CONTRACT TABULATION
Geographic Medicine Branch
Contracts and Agreements
Parasitic Diseases Program

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
NO1 AI 73-2518	Univ. of California, Los Angeles	To develop primary cell lines and to establish permanent cell lines derived from tissues of fresh water molluscs. A variety of media were found to be suitable for the initial establishment of cells in vitro with the addition of calf serum. Growth promoting modifications were the doubling of the strength of the vitamin fraction and increase of calf serum from 8 to 10% per volume.	6/30/72	$ 20,000	Renewal
NO1 AI 72-2527	Oregon State Univ.	To develop primary cell lines and establish permanent cell lines derived from tissues of fresh water molluscs. Primary cell cultures have been routinely established after total tissue dissociation. This approach has made it possible to set up cultures with pre-determined cell densities.	6/27/72	$ 46,736	Renewal
NO1 AI 72-2525	Clinical Pharmacology Research Institute	To develop primary cell lines and to establish permanent cell lines derived from tissues of fresh water molluscs. Techniques have been well developed with embryonic snail tissue. Primary cultures have been established	6/28/72	$ 38,475	Renewal

INDIVIDUAL CONTRACT TABULATION
Geographic Medicine Branch
Contracts and Agreements
Parasitic Diseases Program

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
NO1 AI 72-2525	Continued	and dividing cells have appeared in cultures at 14-36 days, after the majority of primary cells have declined.			
NO1 AI 67-0736	University of Michigan	To supply investigators with animals (Mammals and Snails) infected with various strains of Schistosoma mansoni, S. hematobium and S. japonicum. Approximately 9,000 infected mammals and 5600 infected snails were shipped to qualified investigators in 1973.	4/12/67	$ 67,557	Renewal
NO1 AI 69-0092	University of Georgia	To supply investigators with animals and vectors infected with various filarial species. To test various compounds for chemotherapeutic and/or chemoprophylactic activity against filarial infections. 178 requests for filariasis research materials were filled for 35 investigators representing 29 institutions. 90 carefully selected compounds were tested for chemotherapeutic activity. For the first time, the Brugia Pahangi-Gerbil system was used as a small-animal model for filariasis chemotherapy.	1/22/69	$ 71,851	Renewal

INDIVIDUAL CONTRACT TABULATION
GEOGRAPHIC MEDICINE BRANCH
Contracts and Agreements
Tuberculosis Program

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans for FY 75
NO1 AI 68-0640	Trudeau Institute	Authentication, maintenance and distribution of a mycobacterial culture collection. To supply investigators throughout the world with cultures of biochemically characterized and virulence tested mycobacteria. 876 cultures were distributed. 9 new strains were added to the collection. Growth and storage properties of selected cultures were studied.	2/12/68	$53,757	Indefinite
NO1 AI 70-2079	National Jewish Hospital	To serve as a reference laboratory for the identification of unknown strains of mycobacteria and to provide standard sets of antisera suitable for serotyping certain groups of mycobacteria together with the corresponding reference strains. 302 reference reagents (strains and antisera for serotypes) were distributed. Serotyping procedure for the M. avium-intracellulare-scrofulaceum complex was tested and found to be highly efficient.	2/17/70	39,041	Indefinite

INDIVIDUAL CONTRACT TABULATION
GEOGRAPHIC MEDICINE BRANCH
Contracts and Agreements
Tuberculosis Program

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans for FY 75
NO1 AI 72-2521	Hazleton Laboratories	To study the duration of protection of BCG aerosol vaccination in monkeys. Aerosol BCG vaccination has proved highly effective in the monkey model and has been shown to be dose dependent.	6/28/72	$184,187	Funding covers FY 1974 & 1975
NO1 AI 72-2534	Hazleton Laboratories	To compare the protoplasmic components of mycobacterial cells disrupted by specified sonication procedures and pressure cell techniques. In terms of yield of active components and each of preparation, sonication for 15 minutes appeared to be the most suitable method for preparation of bacillary extracts; however, there were no significant qualitatitive differences in chemical content or biological activity between the preparations even though quantitative differences were noted.	6/28/72	0	Work completed

INDIVIDUAL CONTRACT TABULATION
GEOGRAPHIC MEDICINE BRANCH
Contracts and Agreements
Tuberculosis Program

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans for FY 75
NO1 AI 73-2527	Case Western Reserve	To fractionate and characterize mycobacterial antigens by molecular exclusion and ion exchange chromatography. Approximately 35 fractions will be available this year for detailed skin test reactivity and immulogical testing.	6/29/73	$23,207	Work completed
NO1 AI 73-2526	Eastern Virginia Medical School	To fractionate and characterize mycobacterial antigens by gradient acrylamide gel electrophoresis (GAGE). Approximately 60 fractions will be available this year for detailed skin test reactivity and immunological testing.	11/1/73	36,285	Renewal
NO1 AI 74-C204	Case Western Reserve	To establish the relationships of 002 mycobacterial culture filtrate reference materials to the original 001 reference kit materials. The 002 antiserum was found to be significantly weaker than the 001 serum. Improvement by blending was unsuccessful.	8/23/73	4,985	Work completed in FY 74

INDIVIDUAL CONTRACT TABULATION
GEOGRAPHIC MEDICINE BRANCH
Contracts and Agreements
Tuberculosis Program

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans for FY 75
NO1 AI 74-C513	Case Western Reserve	To develop an immunoelectrophoretic reference pattern and establish the nomenclature for the antigens contained in a mycobacterial cell extract. Work is progressing as scheduled.	2/4/74	$4,978	Work will be completed in FY 75

INDIVIDUAL CONTRACT TABULATION
Geographic Medicine Branch
Contracts and Agreements
Viral Diseases Program

Contract/ Ag. No.	Contractor	Objectives & Findings	Date Initiated	Current Annual Level	Plans For FY 1975
NO1 AI 71-2199	Michigan Department of Public Health	To develop an improved method for inactivation of rabies virus without significant loss of immunogenicity. Six 10 liter lots of rabies vaccines were inactivated with betapropiolactone. This inactivation procedure was based on research conducted during the first two contract years. The inactivated vaccine passed the tests for immunogenicity and safety.	6/29/71	$ 28,000	Renewal
NO1 AI 71-2204	Univ. of Michigan	To isolate and characterize protective antigens of rabies virus. Antigenic components treated with triton were demonstrated to be highly immunogenic. Dissociation of virus particles did not inactivate the protective antigen.	6/29/71	$ 37,215	Renewal
NO1 AI 71-2292	Wistar Institute	To develop simple and economical procedures for the evaluation of the protective acticity of rabies vaccines. Several new methods for measuring the immunological response to rabies were elaborated, including a radioimmune assay for rabies binding antibody, the hemagglutination inhibition test, cytolytic test-releasing radioisotopes from rabies infected cells by means of antibody and complement, and in vitro stimulation of sensitized lymphocytes.	6/1/71	$ 29,000	Renewal

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NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
LABORATORY AND CLINICAL RESEARCH, JULY 1973-JUNE 1974
Report of Scientific Director

The Laboratory and Clinical Research Program continues to be productive as detailed in the individual summaries of the Laboratory Chiefs. A particularly notable event was the first visualization of hepatitis A virus by the technic of immune electron microscopy. Dr. Stephen Feinstone, working with Dr. Albert Kapikian who taught him the technic, and Dr. Robert Purcell who supplied the materials, achieved this as a Research Associate. This work which has since been independently confirmed exemplifies the opportunities open to young post doctoral scientists in the laboratories and opens pathways for the identification of the virus in in vito systems by some mechanism other than use of human volunteers. The accomplishment is singled out because of the long history of frustration of many excellent scientists which preceded it and because of its importance to a major NIAID program area.

The year ended with the disestablishment of the Laboratory of Microbiology as a result of personnel ceiling reductions. Professional staff were reassigned into other laboratories, often with loss of technical support. It was an unpleasant episode that interrupted work by these scientists and which will have the long-range effect of further decreasing their productivity. A small contingent that had been involved in a close collaboration on research involving streptococci were reconstituted into a Laboratory of Streptococcal Diseases under Dr. Roger Cole.

In addition to closing out the Laboratory of Microbiology, the volunteer facility in the Federal Penitentiary at Atlanta, Georgia, was closed. This facility which had been entirely devoted to research on primate malarias, including chemotherapy, has a long and distinguished record. Its closing, while assisting the NIAID to meet its reduced personnel ceiling, was not solely for that reason. Rather, it was a program decision to consolidate all parasitological research at the NIH with a change in direction in malaria research towards the more fundamental processes of host-parasite interactions and mechanisms of resistance. The former primate malaria research section in Chamblee, Georgia, was also closed out and moved to Bethesda. Its staff, with one exception, chose not to accept relocation, and the majority were employed by the Center for Disease Control. The germfree animal unit of the Laboratory of Microbial Immunity was also terminated, the space thereby afforded being important in the consolidation of parasitological research.

The Board of Scientific Counselors met at the Rocky Mountain Laboratory in August 1973. Dr. Robert S. Stone, the recently appointed Director, NIH, attended that meeting. The Board was complimentary on most of the work which was presented to them in some detail. It, however, reiterated previous views that the NIH needed to either increase the number of professional staff, especially in virological research, or to seek some other solution to the problem if this were not possible. These views related to the reductions in staffing which have been necessitated by reductions of the NIAID personnel ceilings, the under-utilization of facilities, and the limited capacity of the NIAID to provide new and vigorous talent to exploit the research on slow viruses, mycobacterial fractions, and other areas.

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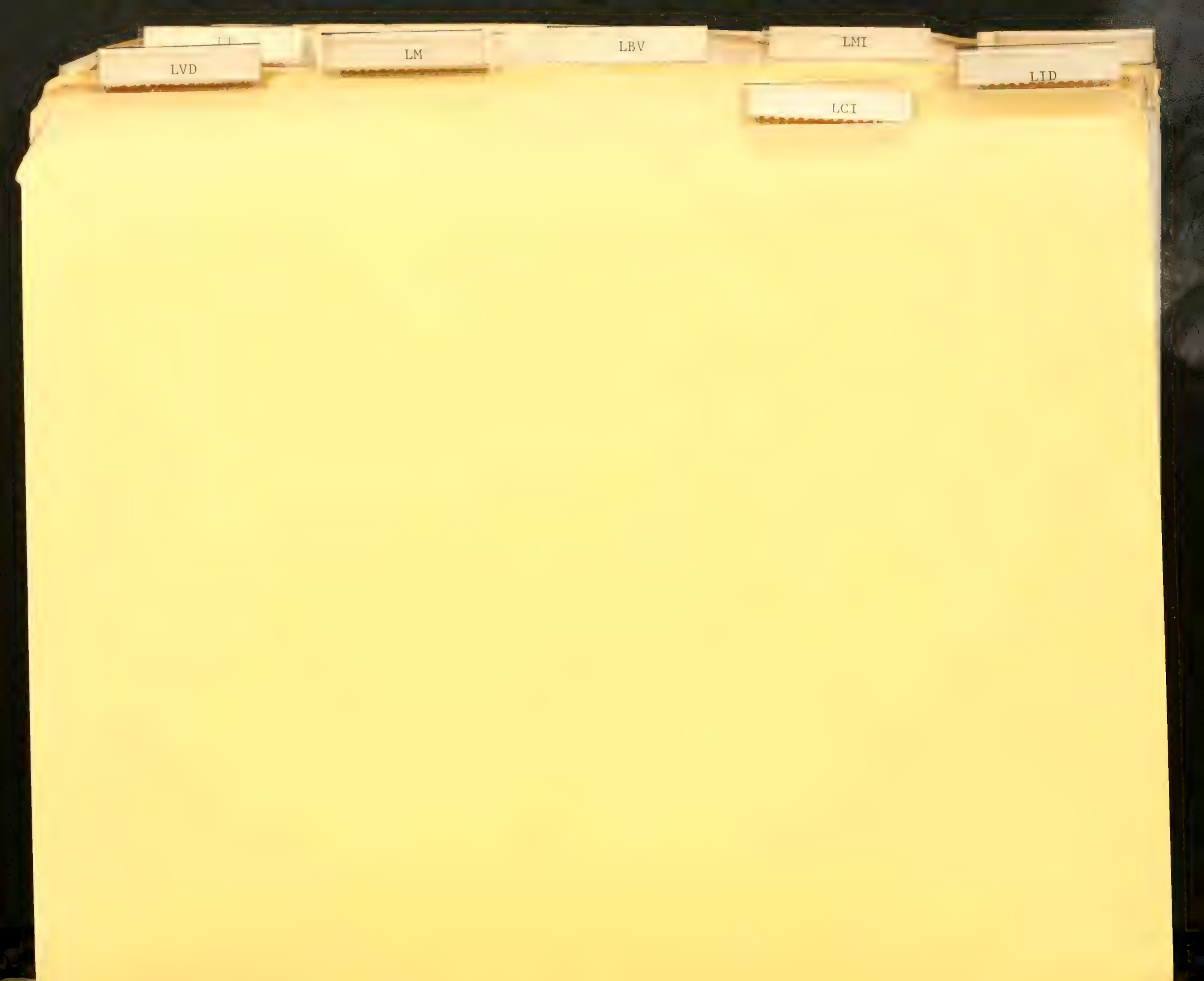
The Board met at the NIH in April, 1974, in the first of a planned two meetings to examine the rationale for NIAID (and NIH) intramural research and discuss future program directions. All Laboratory Chiefs spent the two days of the meeting in discussions with the Board as to the present thrusts and future directions of research in their respective areas, i.e., virology, immunology, bacterial and mycotic diseases, parasitic and vector borne diseases. The discussions were fruitful and laid the groundwork for the Board's further consideration of NIAID program directions in the fall of 1974.

The continuing need for access to healthy volunteers for research on respiratory viruses and acute non-bacterial gastroenteritis led to initiation of a contract for a volunteer facility at the University of Maryland Medical Center in Baltimore. The contract which provides for collaboration in research in volunteers recruited from the community (colleges and other sources) was awarded after a competitive review of these proposals received as a result of advertising. If successful, it will shift such research out of prison populations where much of it has been done in the past. It will expand the capabilities of the normal volunteer program of the Clinical Center for NIAID scientists.

In response to DHEW and NIH desires that programs be better defined in program planning and budgetary presentations, the NIAID has categorized its research into four broad general areas. These are listed along with the percentage of laboratory staff professionals engaged as follows:

Program Area	Number	%
Allergy and Immunologic Diseases	44	26.5
Virus Diseases	58	35.0
Bacterial, Mycoplasmal and Mycotic Diseases	24	14.4
Parasitic and Vector Borne Diseases	40	24.1
TOTAL	166	100.0

This reflects the heavy emphasis in the research program on infectious diseases, particularly virology, over the course of many years. Clinical research in allergy or immediate hypersensitivity has only recently been introduced and the immunological research enhanced largely at the expense of bacteriological and parasitic diseases research. The relatively large percentage of staff devoted in the last area is a result of the inclusion of the acarology and other vector borne disease research staff at the Rocky Mountain Laboratory under this rubric.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LABORATORY OF CLINICAL INVESTIGATION

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Summary of Program
Laboratory of Clinical Investigation
July 1, 1973 - June 30, 1974

Sheldon M. Wolff, M. D., Chief of Laboratory
and Clinical Director, NIAID

Introduction

The past year has been one of continued activity and increasing productivity by the members of the Laboratory of Clinical Investigation. Many notable achievements have been made in a wide variety of areas of clinical investigation. The numbers of individuals from all over the world who have requested training positions, the large number of invitations to participate in important scientific events, the continued increase in numbers and quality of publications all attest to the vitality and substance of the members of the LCI.

Unfortunately, Dr. David C. Dale will be leaving us this summer to accept a senior position in the Department of Medicine of the University of Washington School of Medicine. Dr. Dale's exceptional talents as both a clinician and investigator will be sorely missed by all of us. His departure deprives us of an area of investigation (granulocyte physiology and function) vital to our needs to study the whole host. Because of the personnel situation within the National Institute of Allergy and Infectious Diseases, we shall not be able to recruit an able investigator into this area.

Although Dr. John Gallin will be leaving this year to become Chief Resident in Medicine at New York University School of Medicine, he will return to the permanent staff of the LCI. Dr. Gallin has been exceptionally productive, and we look forward to his return. Dr. Michael A. Kaliner, who trained in the allergy program at the Robert B. Brigham Hospital, will be joining the allergy program of LCI upon completion of his military service in 1975. His addition to our staff will give us considerable strength in the area of immediate hypersensitivity.

The return of Dr. Raphael Dolin this past year has allowed our modest program in virology to begin to grow and become stronger. Dr. Dolin's research into non-bacterial, infectious gastroenteritis has made considerable progress, and he has begun studies on antiviral chemotherapy.

We continue to receive many more qualified applicants for positions in LCI than we have positions. During the past year, postdoctoral trainees from Switzerland, England, and Sierra Leone worked in LCI. Residents from Walter Reed, Bethesda Naval, Georgetown University and George Washington University Hospitals all worked on our wards as did students from many medical schools throughout the U.S.A.

Our patient census continues to run higher than most of the other services at the National Institutes of Health. We have received excellent and valued support from the nursing service and the Clinical Center. Members of LCI continue to play important roles throughout the NIH by serving on the Medical Board, Clinical Research Committee, Radiation Commttee, etc. We are well represented as participants in major medical and scientific meetings all over the world.

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Despite the obvious productivity and success of the members of LCI, we still have the perennial problems of too few positions and not enough space. In order to maintain the quality of staff presently working in LCI, we will need to increase our support personnel and the space available.

Research Accomplishments

The Laboratory continues to place major emphasis on studies relating to clinical immunology, virology, mycology and host defense mechanisms. The role and function of blood cells as major determinants of resistance to infections are extensively investigated.

PATHOPHYSIOLOGY OF HEMOLYTIC ANEMIA

These studies have now defined in an animal model the factors which influence the clearance and destruction of erythrocytes in the circulation. These factors include: the class of antibody which coats the erythrocyte, whether complement is activated, the state of activation of cells of the fixed phagocytic system and the microcirculation in the various organs of the reticuloendothelial system. The studies have examined the function of various agents which are used in the treatment of the disease, autoimmune hemolytic anemia and defined which of the various events in the disease cycle, autoantibody synthesis as opposed to altered cell destruction that they affect. Recent studies have extended these findings to man and it has been possible to show that many of the conclusions derived from animal studies can be directly applied to man. (Atkinson, Frank)

STUDIES ON THE INTERACTION OF ANTIBODY AND COMPLEMENT IN THE PRODUCTION OF IMMUNE DAMAGE

These studies have investigated the interactions between antibody, complement and tissue cells at several levels of relevance to clinical problems. At a practical clinical level, a new sensitive test for the complement component C4 has been developed. The test as developed can be performed in a routine hospital laboratory and, therefore, should make functional assays of this component generally available. New methods for identifying cells in tissue sections have been discovered and these should prove useful in a wide variety of histopathological studies. At a more basic level a number of the factors which allow an invading microorganism to be opsonized have been identified as have some of the receptors on phagocytic cells which allow this opsonization to proceed. Finally, some of the factors which influence C3 turnover and the effects of drugs on the turnover of this component have been examined. (Frank, Gaither)

CLINICAL STUDIES OF COMPLEMENT ABNORMALITIES IN MAN

These studies are helping to define the function of complement in the normal and diseased individual. To that end immunologic function in families with C6 and C2 deficiency have been evaluated. Studies of therapy of hereditary angioedema with EACA and Provera have been continued. Complement component alterations in chronic malaria have been defined and attempts to determine how activation of these components contribute to the manifestations of this disease are being made. (Frank, Neva)

CELL-MEDIATED IMMUNITY

The studies of chronic candidiasis as a model of defective cell-mediated immunity have been extended and now include 29 patients. The prediction that clinical and immunological characterizations would serve to identify various classes of patients has been borne out. Furthermore, these studies have facilitated the selection of treatment schedules. One mode of therapy, restoration of cell-mediated immunity with transfer factor, is apparently beneficial. However,the mechanism of action of transfer factor and the importance of using donors with positive Candida skin tests has not been established. (Kirkpatrick)

Characterization of the physico-chemical properties of transfer factor is underway. Thus far, a unique chemotactic substance in transfer factor has been identified, and some preliminary assays of its composition have been completed. (Kirkpatrick, Gallin)

ANTIGEN RECOGNITION

Antigen recognition by immune lymphocytes requires an obligatory uptake of antigen by macrophages. The handling of soluble protein antigens by macrophages is complex, although a major portion of most (approximately 80%) of macrophage-bound radiolabelled antigen is either released or degraded rapidly (T-1/2 in three to six hours). In view of previous studies this degradative pathway likely occurs via fluid-phase (macro) pinocytosis. A second functional pathway is not degradative and may occur via an adherence dependent pathway (micropinocytosis). This latter pool is stable for up to 72 hours with respect to TCA and antibody precipitability and is not present on the surface membrane as judged by binding of specifically purified FAB'_2 anti-antigen or by proteolytic enzyme susceptibility. Moreover, anti-antigen does not inhibit T lymphocyte recognition of macrophage-bound antigen.

Recognition of macrophage-bound antigen precedes via an initial reversible antigen independent binding of lymphocyte to the macrophage. This event requires active macrophage but not lymphocyte metabolism. This initial antigen-independent binding is followed by a period of antigen dependent binding and cell division if and only if:1) both macrophage and lymphocyte share identity of membrane H-linked determinants, and 2) the macrophage carries the appropriate antigen for which the lymphocyte bears an immunospecific receptor. Thus, antigen-independent binding would serve to approximate or focus T or B lymphocytes on macrophages without conveying immunological specificity; a subsequent antigen and histocompatibility dependent physical and functional phase of macrophage-lymphocyte interaction would serve to convey specificity and facilitate regulation of genetically controlled immune responses. Further studies to evaluate this model are in progress. (Rosenthal, Lipsky, Ellner, Greineder, Blake, Cehrs).

KININS

Studies of the mechanism of generation of bradykinin in human plasma and the interrelationship of this pathway with the coagulation, fibrinolytic and complement pathways are being investigated. Further progress has been made in preparing large quantities of unactivated Hageman factor and its substrates,

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pre-PTA, prekallikrein, and plasminogen proactivator from human plasma. Pre-PTA has been totally purified from plasma by chromatography on QAE Sephadex, SP Sephadex, Sephadex G-150 and passage over an anti-IgG immunoabsorbant. It was found to have a molecular weight of 165,000-175,000 and an isoelectric point of 9.0-9.1. It is activated enzymatically by Hageman factor or Hageman factor fragments. The major plasma inhibitor of activated PTA appears to be α_1 antitrypsin. However, C1 INH and antithrombin III also contribute significantly to its destruction. (Kaplan, Heck)

Kallikrein and plasminogen activator were found to be chemotactic for human mononuclear cells in addition to their previously described activity upon neutrophils. This chemotactic activity was dependent upon the integrity of the active site of each enzyme and the natural inhibitors of each enzyme were shown to also function as chemotactic inhibitors. Thus, α_2 macroglobulin inhibits the chemotactic activity of both kallikrein and plasminogen activator whereas C1 INH inhibits only kallikrein. Activation of these factors has been shown to be initiated both *in vitro* and *in vivo* by highly purified bacterial endotoxin. (Kaplan, Gallin)

The Fletcher factor deficiency was further evaluated and was found to be secondary to prekallikrein deficiency. Thus, this plasma generates no bradykinin; however, its coagulation and fibrinolytic defects are caused by absence of a feedback mechanism in which kallikrein activates Hageman factor. However, although activated Hageman factor corrects all of the defects of this plasma (except kinin generation), reconstitution with kallikrein in the absence of kaolin does not recruit sufficient Hageman factor to correct the coagulation and fibrinolytic defects, suggesting that Hageman factor and kallikrein activate each other enzymatically on the surface but that initiation by the surface is solely dependent upon Hageman factor. (Kaplan)

Cold urticaria has been found to be IgE mediated in most patients. The symptoms of itching and swelling in response to cold has been shown to be mediated largely by histamine release. This was concluded after serial determination of plasma histamine levels by the radioenzyme method in patients challenged with a cold stimulus. The sensitivity can be passively transferred to normal volunteers by a standard Prausnitz-Kaustner (PK) test; the activity of the patients' plasma can be removed by passage over an anti-IgE immunoabsorbant. The eluted IgE is being studied in terms of the effect of temperature upon its interaction with human basophils and mast cells. (Kaplan)

PULMONARY HOST DEFENSES

The general area of pulmonary host defenses and nosocomial infections is being investigated, using *Pseudomonas* pneumonia as the prototype infection because of its frequent occurrence in immunosuppressed patients who are most susceptible to opportunistic microorganisms. Initial work has been done in rabbits to explore ways of manipulating antibody production in the respiratory tract and to assess the effectiveness of these antibodies in promoting phagocytosis and killing by alveolar macrophages. IgG was found to be the principal opsonin in the lung; maximal amounts of IgG opsonic antibody could be produced with a combination of intramuscular and intranasal (local secretory IgG produced) immunization. IgA antibody was induced with intranasal vaccination but was

judged to have less opsonic activity. Competent phagocytic cells are essential for the pulmonary defenses. In addition, intranasal immunization with Pseudomonas lipopolysaccharide sensitized respiratory lymphocytes which could be induced to release migration inhibition factor, which is an *in vitro* correlate of delayed hypersensitivity or cellular immunity. However, evidence of cellular immunity in the respiratory tract was transient following primary immunization and persisted only about three weeks. Thus, the importance of cellular immunity in the coordinated host defense of the lung against Pseudomonas infection needs to be assessed further. (Reynolds)

ANTIBIOTIC CONCENTRATION IN THE LUNG

Frequently, antibiotic therapy is ineffective in severe pulmonary infections such as *Pseudomonas* pneumonia becuase adequate concentrations of the drugs are not obtained in the lung or respiratory secretions. Relatively little information is available about the penetration of antibiotics into the lung secretions or about the clearance from them. Studies in a dog model with gentamicin, tobramycin, and carbenicillin have shown that maximal concentrations of these drugs in bronchial secretions range from 15-30% of maximal serum values and that 30-50% of *Pseudomonas aeruginosa* isolates may not be adequately inhibited by these levels of antibiotics. However, the clearance of antibiotics from the bronchial secretions is relatively prolonged compared with serum (T-1/2 in bronchial secretion is two to three times longer), suggesting that the effective concentrations in the bronchial secretions may be longer, if adequate levels could be achieved. (Reynolds, Pennington)

SECRETORY IMMUNOGLOBULINS

The ultrastructure of canine secretory component and J-chain are being investigated now that they have been isolated and purified from secretory IgA; the combination of these chains with 7S IgA monomer may elucidate the assembly of the secretory IgA molecule. (Reynolds)

PEYER'S PATCH LYMPHOCYTES

While investigating the origin of precursor cells that differentiate into immunoglobulin secreting plasma lymphocyte cells in the gastrointestinal tract, the interactions of orally administered antigens and processing cells (T-lymphocytes) in gut-associated lymphoid tissue (Peyer's patches) were studied in adult guinea pigs. A detailed identification of the lymphoid cell populations in Peyer's patches was made. (Reynolds, Levin)

MECHANISM OF IMMUNOSUPPRESSION

Studies of the specific effects of immunosuppressive agents in man have shown that corticosteroid-induced lymphocytopenia results not from a destruction of circulating lymphocytes, but from a redistribution of these long-lived recirculating thymus-derived lymphocytes to other body compartments. It was demonstrated in the guinea pig (which is similar to man in its sensitivity to corticosteroids) that this redistribution is to the bone marrow. Thus, corticosteroids enhance the immunocompetence of the bone marrow while selectively depleting peripheral blood lymphocyte subpopulations. Patients on alternate-day prednisone therapy have a cyclic time and dose-related

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transient lymphocytopenia and monocytopenia with every two-day cycle of drug administration. These effects are not cumulative and immunological function is normal except for a few hours following the drug. This explains the intact delayed hypersensitivity responses and normal host defenses in these patients. (Fauci)

Animal model studies have shown that corticosteroid-induced immunosuppression is due to a redistribution of circulating lymphocytes, as well as an interference with the effector limb of the immune response. Cytotoxic agents such as cyclophosphamide and azathioprine have complex mechanisms of action and affect lymphocyte subpopulations in a divergent manner explaining the varying effectiveness of these different agents in certain diseases. (Fauci, Balow, Hurley)

GRANULOCYTES

Studies of cyclic neutropenia indicate that there is cyclic hematopoiesis in this disease with cyclic changes of erythropoietin and colony stimulating factor. The neutrophils in canine cyclic neutropenia are defective. The canine disease can be cured by marrow transplantation. Prednisone causes prolongation of the neutrophil half-life. With alternate-day prednisone, the day off therapy neutrophil kinetics are normal. This form of therapy is not accompanied by any increased susceptibility to infections. (Dale, Chusid)

FEVER

Human leukocytic pyrogen has been found to be at least two distinct molecules, one derived from monocytes and the other from granulocytes. Both are proteins but the monocyte pyrogen has a molecular weight of 38,000 and an isoelectric point of 5.1, while the neutrophil pyrogen has a molecular weight of 15,000 and an isoelectric point of 6.9. There are other biologic and chemical differences as well. (Dinarello, Wolff)

FAMILIAL MEDITERRANEAN FEVER

A controlled, double-blind trial has demonstrated, for the first time, that colchicine is an effective prophylactic agent in the prevention of acute attacks of familial Mediterranean fever. (Dinarello, Alling, Dale, Wolff)

NONSPECIFIC RESISTANCE

A water soluble material derived from cultures of M. smegmatis has been shown to be effective in inducing resistance to a wide variety of infections in mice. Other studies have demonstrated that endotoxin and etiocholanolone both have striking effects on plasma iron and feritin in human beings. (Elin, Wolff)

VIRAL GASTROENTERITIS

The antigenic relationships between three viral agents of gastroenteritis (Norwalk, Hawaii, and Montgomery County) were studied by cross challenge in volunteers and by immune electron microscopy (IEM). Norwalk and Hawaii appear to be antigenically distinct, while Norwalk and Montgomery County appear to be

related. Employing IEM, a viral particle of possible etiologic significance was visualized in stool filtrates from volunteers infected with the Hawaii agent. The response of volunteers experimentally infected with Hawaii virus was also examined. Per-oral intestinal biopsies revealed jejunal histopathologic changes confined to the mucosa during acute illness, with apparent sparing of the large intestine. These reversible changes are similar to those previously described for the Norwalk agent, and may represent the general lesion of viral gastroenteritis. PHA stimulation of peripheral lymphocytes was depressed during acute gastroenteritis through a non-lymphopenic mechanism. (Dolin)

HERPES VIRUS INFECTIONS

A study of the efficacy of adenine-arabinoside (ARA-A) in the treatment of herpes simplex and herpes zoster infections in the immunosuppressed, along with herpes simplex encephalitis in adults and neo-nates, was begun, in a randomized, double-blind, controlled trial. A rapid, sensitive *in vitro* assay of chemical anti-viral activity was established, which may prove clinically useful in measuring levels of drugs in body fluids and tissues. Mean concentrations of ARA-A and IUDR required to inhibit growth of herpes simplex, herpes zoster, cytomegalovirus and EBV were determined. IUDR and ARA-A appeared to have synergistic activity against Herpes Simplex types I and II *in vitro*. Techniques for assaying the effect of anti-viral substances on viral growth in human fetal intestinal and tracheal organ cultures were developed. (Dolin)

PARVOVIRUSES (ADENOVIRUS-ASSOCIATED VIRUSES) OF MAN

The adenovirus-associated viruses (AAV) are defective viruses, which commonly infect man, but have not been associated with disease. Herpes viruses, including oncogenic viruses of primates, EBV and H. Saimiri, provided incomplete helper functions for AAV. Kinetic studies suggested that AAV nucleic acid replication is closely linked to adenoviral nucleic acid replication. Conditional mutants of adenovirus are being employed to further study this interaction. (Dolin)

HISTOPLASMOSIS

The causative fungi for American histoplasmosis (*Histoplasma capsulatum*) and African histoplasmosis (*H. duboisii*) have long been considered as two different species on the basis of their clinical manifestations and the size of their yeast cells in tissue. Discovery of mating system in *H. capsulatum* at our laboratory made it possible to determine the relationship of the two fungi. Eighteen clinical isolates from cases of African histoplasmosis successfully mated with isolates from cases of American histoplasmosis. This is definitive evidence that the two fungi belong to the same species. The test also indicated that "-" type is considerably more frequent than "+" type in African histoplasmosis, consistant with our findings with American histoplasmosis. (Kwon-Chung)

ASPERGILLOSIS

For the first time, resistance to nystatin and amphotericin B in a filamentous fungus was shown to be genetically controlled. Using strains of Aspergillus fennelliae genetic analyses showed that the sensitivity to the drug is directly related to the cell content of ergosterol. Also ergosterol synthesis was directly related to production of sexual and asexual reproduction. (Kwon-Chung)

CRYPTOCOCCUS NEOFORMANS

Human peripheral blood mononuclear cells have been found able to kill the encapsulated fungus, Cryptococcus neoformans, by an antibody dependent, non-phagocytic mechanism. Tissue cells have been known to be destroyed by this mechanism but this appears to be the first description of a microorganism as the target cell. (Bennett)

Importance of the alternate complement pathway in phagocytosis of cryptococci has been underscored by the discovery that antibody-coated cryptococci are poor receptors for early components of the classical pathway. (Bennett, Frank)

PARASITIC DISEASES

Gradually increasing numbers of referrals for questions of diagnosis of parasitic infection are being seen. The malaria complement studies in collaboration with Drs. Frank and Atkinson have been written up for publication and are cited under a different project no. (134, LPD) as are the studies of Dr. Nash (No. 121-K, LPD). Most of the effort during the past year for clinical studies went into a leishmaniasis case in a Peace Corps volunteer from Senegal, Africa. His organism has been established in a mouse model which promises to be very useful for assessment of immunology in this disease and drug treatment. The patient was resistant to therapy in spite of having apparently normal cell-mediated immune responses. Collaborative studies on filariasis are being planned as an extension of work started by Drs. Kaplan of LCI and Pacheco of LPD on IgE in uses of eosinophilic lung. (Neva, Nash)

CHEMOTAXIS

Clinical studies of leukocyte chemotaxis in patients with increased susceptibility to infections have demonstrated defective mononuclear cell chemotaxis in patients with the Chediak-Higashi syndrome as well as in mink and cattle with a similar disease. Abnormal mononuclear cell chemotaxis was also noted in a patient with mucocutaneous candidiasis, a neutrophil chemotactic defect and impaired cellular immunity. Defective neutrophil chemotaxis was noted in two patients with hypogammaglobulinemia. (Gallin)

Studies of normal neutrophil-chemotactic factor interaction demonstrated that decreases in surface charge, calcium release from neutrophils and microtubule assembly is associated with a normal chemotactic response. In vivo generation of chemotactic factors (products of the complement, kinin-generating and fibrinolytic systems) were demonstrated in normal volunteers given endotoxin. Studies of neutrophil phagocytosis demonstrated neutrophil secretory products

capable of activating the complement system as well as directly cleaving the chemotactic factor C5a from C5; an inactivator of C5a was also demonstrated to be secreted from neutrophils during phagocytosis. A new chemotactic factor in dialyzable transfer factor has been described. (Wright, Gallin)

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Serial No. NIAID-10(c)
1. LCI
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Office of the Chief

Previous Serial Number: SAME

Principal Investigator: Dr. Sheldon M. Wolff

Other Investigators: NONE

Cooperating Units: NONE

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	3/12			3/12
Professional	3/12			3/12
Other				

Project Description:

Objectives:

To develop, direct and coordinate the program of the Laboratory, as defined in the individual research projects; where necessary, to redirect individual projects to meet current needs and advances in the field; to align clinical projects with patient availability; to provide an unexcelled standard of patient care for patients utilized in research.

Methods Employed:

Organize available staff and recruit qualified personnel, both professional and sub-professional; to develop the program and carry out its aims; maintain close liaison with area medical societies, institutions and individuals for referral of patients whose diagnoses fall within the active or proposed disease research categories; the highest standards of patient care are maintained by selection of qualified physicians; the development of necessary

policies and continued close and direct supervision of patient care activities: professional consultant services to other institutes in the area of infectious diseases; continued guidance of research projects undertaken by younger investigators; maintenance of staff morale.

Proposed Course: To continue.

Publications:

1. Northrup, R. S. and Chisari, F. V.: Cholera toxoid reactions in human volunteers. Clinical and histological studies. Progr. Immunobiol. Standard. 5: 355-364, 1972.

2. Kasel, J. A., Couch, R. B., Gerin, J. L., and Schulman, J. L.: Effect of influenza antineuraminidase antibody on virus neutralization. Infect. Immun. 8: 130-131, 1973.

3. Couch, R. B., Kasel, J. A., Pereira, H. G., Haase, A. T., and Knight, V.: Induction of immunity in man by crystalline adenovirus type 5 capsid antigens. Proc. Soc. Exp. Biol. Med. 143: 905-910, 1973.

4. Bujak, J. S., Ottesen, E. A., Dinarello, C. A., and Brenner, V. J.: Nocardiosis in a child with chronic granulomatous disease. J. Pediatr. 83: 98-100, 1973.

5. Sheagren, J. N., Simon, H. B., and Rich, R. R.: Therapy of sarcoidosis initiated with alternate-day prednisone. J. Natl. Med. Assoc. 65: 391-398, 1973.

6. Murphy, B. R., Chalhub, E. G., Nusinoff, S. R., Kasel, J., and Chanock, R. M.: Temperature-sensitive mutants of influenza virus. III. Further characterization of the ts-1[E] influenza A recombinant (H_3N_2) virus in man. J. Infect. Dis. 128: 479-487, 1973.

Serial No. NIAID-11(c)
1. LCI
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Clinical Care Program in Support of Research Projects

Previous Serial Number: SAME

Principal Investigators: Dr. Sheldon M. Wolff
Dr. Jerrold J. Ellner

Other Investigators: Dr. Cecil Bourne
Dr. Oscar A. Cartaya
Dr. Richard A. Clark
Dr. Jeffrey A. Gelfand
Dr. Fred A. Gill
Dr. David T. Greenberg
Dr. Charles J. Jaffe
Dr. Bernard S. Jay
Dr. John A. Kazmierowski
Dr. Bernard M. Kruger
Dr. Susan G. Larson
Dr. Donald R. MacCaffree
Dr. Michael Monoit
Dr. Peter G. Sohnle
Dr. Paul C. Turkeltaub
Dr. Daniel G. Wright
Dr. Lewis D. Yecies

Cooperating Units: NONE

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	4-5/12		1-9/12	6-2/12
Professional	4-5/12		1-9/12	6-2/12
Other				

Project Description:

Responsibility for care of patients in the various research areas.

Proposed Course: To continue.

Publications: NONE

Serial No. NIAID-12(c)
1. LCI
2. Clinical Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Mechanisms of Fever and Host Responses

Previous Serial Number: SAME

Principal Investigator: Dr. Sheldon M. Wolff

Other Investigators: Dr. David C. Dale
Dr. Charles A. Dinarello
Dr. Anthony S. Fauci
Dr. Michael M. Frank
Dr. John I. Gallin
Dr. Allen P. Kaplan
Dr. Charles H. Kirkpatrick
Dr. Alan S. Rosenthal
Dr. Daniel G. Wright
Mr. Joseph B. Edelin
Mr. Stanley B. Ward

Cooperating Units: Dr. David W. Alling, OSD, NIAID, NIH (no project no.)
Dr. Warner Barth, Washington Hospital Center, Washington, D. C.
Dr. Benjamin E. Cohen, Massachusetts General Hospital, Boston, Massachusetts
Dr. John R. Durocher, Walter Reed Army Institute of Research, Washington, D. C.
Dr. Ronald J. Elin, CPD, CC, NIH (no project no.)
Dr. Clement Finch, Department of Hematology, University of Washington Medical Center, Seattle, Washington
Dr. Ira Goldstein, New York University School of Medicine, New York, New York
Dr. Harvey R. Gralnick, CPD, CC, NIH (no project no.)
Dr. Herbert D. Hochstein, LCA, BB, NIH (no project no.)
Dr. Robert Horn, Vanderbilt University School of Medicine, Department of Pathology, Nashville, Tennessee
Dr. Ralph E. Johnson, DCBD, NCI, NIH (no project no.)
Dr. Arthur S. Levine, DCT, NCI, NIH (project no. NCI-6830)
Dr. George A. Padgett, Washington State University, Pullman, Washington
Dr. Seymour Perry, DCT, NCI, NIH (no project no.)
Dr. Elliot Vesell, M. S. Hershey Medical Center, Department of Pharmacology, Hershey, Pennsylvania

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	4-11/12			4-11/12
Professional	2-11/12			2-11/12
Other	2			2

Project Description:

Selected patients are being studied on the wards and in the laboratory. The patients can be divided into the following groups: patients with a) recurrent fever; b) Chediak-Higashi syndrome; c) granulomatous disorders; d) cyclic and other neutropenias; e) eosinophilia; and f) hypersensitivity states; and normal volunteers.

Extensive studies are in progress relating to the pathogenesis of fever, granulocyte function, immune and other responses of humans and experimental animals to infections, antigens, etc.

Objectives:

1. To assess varied biologic responses of normal and a variety of patients to pyrogens.

2. To assess the responses of humans to multiple doses of endotoxin (endotoxin tolerance).

3. To intensively study groups of patients with such poorly defined illnesses as familial Mediterranean fever, recurrent fever, Chediak-Higashi syndrome, midline granuloma, Wegener's granulomatosis, cyclic and other neutropenias in an attempt to better delineate the clinical syndrome.

4. To study the mechanisms responsible for the release of endogenous pyrogen.

5. To characterize and purify leukocytic pyrogen.

6. To assess the effect of immunosuppressive agents on human diseases.

7. To explore the pathogenesis of the increased susceptibility to infection in a variety of diseases.

8. To determine the marrow granulocyte reserves in neutropenic states and to perfect better tests for this parameter.

9. To study the effect of vitamin A on infection in mice.

10. To study the effect of fever on drug metabolism.

11. To determine the effect of endotoxin and etiocholanolone on coagulation and iron metabolism in man.

12. To define the features of the beige mouse which are similar to the Chediak-Higashi syndrome of man.

13. To determine the susceptibility of beige mice to infection.

14. To assess the protective effects of endotoxin in mice challenged with various mycotic organisms and, if possible, to characterize the humoral and cellular factor(s) responsible for protection.

15. To evaluate the specificity of limulus amebocyte lysate for compounds other than endotoxin.

16. To evaluate the utility of the limulus amebocyte lysate test to detect endotoxemia in man.

17. To characterize the humoral factors responsible for endotoxin tolerance.

18. To investigate the growth of C. albicans in human sera.

19. To identify patients with abnormal neutrophil and/or mononuclear cell chemotaxis and patients with deficiencies in the generation of chemotactic factors.

20. To continue studies of calcium fluxes during chemotaxis and evaluate the role of microtubules in the chemotactic response.

21. To study the surface charge of human neutrophils after exposure to chemotactic factors.

22. To evaluate the chemotactic properties of products of phagocytosis.

23. To continue studies on the chemotactic activity in dialyzable transfer factor.

24. To study the effect of in vivo endotoxin on the in vitro chemotactic activity of human plasma.

25. To continue studies on the chemotactic activity of products of the fibrinolytic and kinin-generating system.

Methods Employed:

Patients and volunteers receive varying doses of endotoxin and etiocholanolone. Rectal temperatures are taken and a variety of responses are determined: antibody levels, immunoglobulins, plasma cortisol, growth hormone, serum iron, clotting factors, etc.

The mobilization of peripheral granulocytes in response to etiocholanolone, endotoxin, or cortisol is studied in patients before, during and after chemotherapy. Changes in serum iron are determined by standard methods.

Microorganisms were grown in appropriate media, quantitated by turbidity and intravenously injected into mice.

Limulus amebocyte lysate was prepared from limulus crabs. The lysate was used to study endotoxemia in man and specificity of the reaction.

Mice are given endotoxin and subsequently challenged at varying time intervals with such infectious agents as Candida albicans, Plasmodium berghei, etc. Donor mice are exsanguinated; the whole sera or fractions are passively transferred to normal mice, which are then challenged with the infectious agent. Iron is given to mice to study its protective role against infections.

Human peripheral leukocytes are incubated overnight with killed S. albus organisms. The resulting supernatant contains leukocytic pyrogen. This material is concentrated by means of alcohol precipitation, dialysis, column chromatography and fractionation, isoelectric focusing and electrophoresis.

Peripheral blood leukocytes were separated from heparinized whole blood by dextran sedimentation or Hypaque-Ficoll separation. Neutrophil chemotaxis was evaluated using a radioassay employing ^{51}Cr-labeled leukocytes and a double micropore filter Boyden chamber previously developed in our laboratory. Mononuclear cell chemotaxis was performed with a morphologic assay. Chemotactic factors were generated by incubating serum with either endotoxins or antigen-antibody complexes (complement related factors) or with kaolin (kinin related factors). The chemotactic factors were then purified by sephadex chromatography.

Calcium flux studies were performed using ^{45}Ca. Leukocyte microtubules were studied morphologically with an electron microscope.

Phagocytosis was studied using Hypaque-Ficoll purified neutrophils and latex particles were used for the phagocytic challenge.

Patient Material:

Patients are admitted to the Clinical Center, as are normal volunteers, for these studies. In addition, patients from other Institutes participate in these studies.

Major Findings:

Patients with recurrent fever of unknown etiology have been found to represent a diverse array of clinical disorders.

Human leukocytic pyrogen has been found to be at least two molecules, one from polymorphonuclear leukocytes and the other from monocytes.

Cyclophosphamide has produced striking remissions and clinical improvement in patients with Wegener's granulomatosis, nephrosis and a variety of hypersensitivity diseases.

Poly (I)·poly (C), poly (A)·poly (U), ribonuclease, thrombin and thromboplastin were found to effect a positive limulus test.

In vitro and *in vivo* studies demonstrated that changes in iron metabolism induced by bacterial endotoxin are a key factor in the mechanism of resistance to *C. albicans* infection in mice.

The Chediak-Higashi mice had a significant increased susceptibility to infection with *E. coli*, *K. pneumoniae*, *S. aureus*, *S. pneumoniae* and *C. albicans*. Serum immunoglobulin concentrations (IgG_1, IgG_2, IgA and IgM) were essentially the same in both groups of mice.

Those volunteers who achieved a significant increase in fever after etiocholanolone injection showed a significant prolongation of their plasma antipyrine half-life compared with the plasma antipyrine half-life during an afebrile control period.

Vitamin A was found to induce nonspecific resistance to infection in mice using *P. aeruginosa*, *L. monocytogenes* and *C. albicans* as the challenge organisms.

Defective mononuclear cell chemotaxis was demonstrated in patients with the Chediak-Higashi syndrome and in mink and cattle with a similar disease using the morphologic and a new ^{51}Cr radioassay of mononuclear cell chemotaxis. Abnormal mononuclear cell chemotaxis was also demonstrated in a patient with previously described depressed neutrophil locomotion. Defective neutrophil chemotaxis was found in two patients with hypogammaglobulinemia. Impaired generation of complement related chemotactic factors (C5a) was demonstrated in serum from a patient deficient in the "r" fragment of the first component of complement and deficient chemotactic factor activity was noted in plasma from patients deficient in preKallikrein (no kallikrein) or Hageman factor (decreased kallikrein and plasminogen activator).

An association between chemotaxis, calcium release from neutrophils and microtubule assembly was demonstrated. Such microtubule assembly may be important in establishing the net vector of locomotion required for a normal chemotactic response.

Interaction of neutrophils with four different chemotactic factors resulted in a decrease in the net negative surface charge of the cell. This decrease in surface charge was not associated with sialic acid release and was not seen with nonchemotactic proteins. These results may represent the binding of chemotactic factors to negatively charged membrane receptors or may represent membrane surface changes associated with the normal chemotactic response. Pretreatment of cells with steroids prevented the chemotactic factor induced changes in surface charge.

Phagocytizing neutrophils were found to release an enzyme that directly cleaves the chemotactically active fragment C5a from C5 and also activates the complement system in serum. Also released by phagocytizing neutrophils was a C5a inactivator. These secretory products of phagocytosis may be important in regulating inflammatory-immune responses.

Additional studies on the chemotactic activity in dialyzable transfer factor demonstrated this material has in vivo (monkeys) as well as in vitro chemotactic activity. More refined preparative techniques have not resulted in the separation of the chemotactic and transfer factor activities and suggests that their activities are associated with the same molecule.

In vivo administration of endotoxin to normal human subjects resulted in the in vivo generation of chemotactic activity in about 50% of volunteers. Sephadex chromatography of plasma obtained after administration of endotoxin demonstrated this activity resulted from activation of the kinin generating and fibrinolytic pathways. Two previously undescribed chemotactic molecules, one with a molecular weight about the same as albumin and one with a molecular weight about the same as histamine were also noted. These latter two molecules were also generated in vitro by kaolin activation of normal serum; kaolin activation of serum deficient in prekallikrein or Hageman factor lacked these molecules, suggesting that they result from Hageman factor activation.

Kallikrein and plasminogen activator were found to have potent chemotactic activity for human mononuclear cells. The naturally occurring plasma inhibitors of kallikrein and plasminogen activator were shown to inhibit their chemotactic activity.

Significance to Biomedical Research and the Program of the Institute:

The large number and wide variety of studies listed above provide considerable new knowledge dealing with host response mechanisms to a wide variety of clinical and experimental diseases and the pathogenesis of fever.

These studies provide insight into the mechanisms of nonspecific resistance to infection. After additional studies, a compound such as Vitamin A may prove to enhance resistance to infection in man. The alteration of a drug antipyrine metabolized by the liver during fever has clear clinical implications. Defining the specificity and properties of the limulus amebocyte lysate test for detecting bacterial endotoxin clinically and experimentally is important.

The clinical studies clearly demonstrate that chemotaxis is an important host defense. The demonstration of *in vivo* generation of chemotactic factors in normal volunteers provides important *in vivo* correlates to *in vitro* observations. A major remaining question is,"What is the mechanism by which leukocytes respond to a chemical signal to move in a directed fashion, and what are the cellular abnormalities in patients with defective chemotaxis?" The studies of calcium fluxes, microtubule assembly and surface charge changes provide initial attempts at characterizing the mechanism of the normal cellular response and will be extended to the pathological conditions. The studies on the chemotactic activity in dialyzable transfer factor and the ability of products of phagocytosis to recruit complement related chemotactic factors and to also inactivate them provides further mechanisms by which inflammatory and immune responses may be related and regulated *in vivo*.

Proposed Course:

The above outlined studies are to be continued and some areas are to be expanded.

Honors and Awards:

Dr. Gallin was invited to be a participant in the Symposium on the Phagocytic Cell in Host Resistance, in Winter Park, Florida, March 5-8, 1974.

Dr. Wolff:

1. Consultant (immunology) in Brazil, Pan American Health Organization, 1973.

2. Consultant, Federal University of Rio de Janeiro, 1973.

3. Member, Editorial Board, the Journal of Clinical Investigation, 1974 (for a five-year term).

4. Served as Visiting Professor or lecturer at many institutions including the University of Virginia, Charlottesville, Va.; Wayne State University, Detroit, Michigan; Massachusetts General Hospital, Tufts University, Boston, Massachusetts; George Washington University, Howard University, and Georgetown University, Washington, D. C.; New Jersey College of Medicine, Newark, New Jersey; Emory University, Atlanta, Georgia; and Albert Einstein College of Medicine, New York, New York.

Publications:

1. Kimball, H. R., Wolff, S. M., Talal, N., Plotz, P. H., and Decker, J. L.: Marrow granulocyte reserves in the rheumatic diseases. Arthritis Rheum. 16: 345-352, 1973.

2. Kass, E. H. and Wolff, S. M. (Eds.): Bacterial Lipopolysaccharides. Chemistry, Biology and Clinical Significance of Endotoxins. Supplement. J. Infect. Dis., Vol. 128, 1973, pp. S1-S305.

3. Aptekar, R. G., Atkinson, J. P., Decker, J. L., Wolff, S. M., and Chu, E. W.: Bladder toxicity with chronic oral cyclophosphamide therapy in non-malignant disease. Arthritis Rheum. 16: 461-467, 1973.

4. Aptekar, R. G., Decker, J. L., Bujak, J. S., and Wolff, S. M.: Adult onset juvenile rheumatoid arthritis. Arthritis Rheum. 16: 715-718, 1973.

5. Elin, R. J.: Erythrocyte survival in magnesium deficient rats. Proc. Soc. Exp. Biol. Med. 142: 1159-1161, 1973.

6. Dinarello, C. A., Ward, S. B., and Wolff, S. M.: Pyrogenic properties of bleomycin (NSC-125066). Cancer Chemother. Rep. 57: 393-398, 1973.

7. Bujak, J. S., Aptekar, R. G., Decker, J. L., and Wolff, S. M.: Juvenile rheumatoid arthritis presenting in the adult as fever of unknown origin. Medicine 52: 431-444, 1973.

8. Elin, R. J. and Wolff, S. M.: Effect of endotoxin administration on the growth of Candida albicans in sera from various species. Can. J. Microbiol. 19: 639-641, 1973.

9. Elin, R. J. and Wolff, S. M.: Bacterial endotoxins. In Laskin, A. I. and Lechevalier, H. A. (Eds.): CRC Handbook of Microbiology. Cleveland, Ohio, CRC Press, Vol. II, 1973, pp. 215-239.

10. Elin, R. J.: A simple method for determining erythrocyte survival in rats using ^{51}chromium. Lab. Animal Sci. 23: 653-656, 1973.

11. Elin, R. J. and Wolff, S. M.: Nonspecificity of the limulus amebocyte lysate test: positive reactions with polynucleotides and protein. J. Infect. Dis. 128: 349-352, 1973.

12. Elin, R. J. and Wolff, S. M.: Endotoxin-induced non-specific resistance to infection--a possible mechanism. In Braun, W. and Ungar, J. (Eds.): "Non-Specific" Factors Influencing Host Resistance. Basel, S. Karger, 1973, pp. 371-381.

13. Elin, R. J. and Wolff, S. M.: Effect of pH and iron concentration on growth of Candida albicans in human serum. J. Infect. Dis. 127: 705-708, 1973.

14. Kimball, H. R., Wolff, S. M., Talal, N., Plotz, P. H., and Decker, J. L.: Marrow granulocyte reserves in the rheumatic diseases. Arthritis Rheum. 16: 345-352, 1973.

15. Hochstein, H. D., Elin, R. J., Cooper, J. F., Seligmann, E. B., Jr., and Wolff, S. M.: Further developments of limulus amebocyte lysate test. Bull. Parenteral Drug Assoc. 27: 139-148, 1973.

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16. Goldstein, I., Hoffstein, S., Gallin, J., and Weissmann, G.: Mechanisms of lysosomal enzyme release from human leukocytes: microtubule assembly and membrane fusion induced by a component of complement (C5a/chemotaxis/cytochalasin B/cAMP: cGMP antagonism). Proc. Natl. Acad. Sci. USA 70: 2916-2920, 1973.

17. Wolff, S. M. and Simon, H. B.: Granulomatous hepatitis and prolonged fever of unknown origin. Trans. Am. Clin. Climatol. Assoc. 84: 149-165, 1973.

18. Wolff, S. M., Kimball, H. R., and Marshall, J. R.: The effects of hydrocortisone and estrogen on experimental fever induced by etiocholanolone. J. Infect. Dis. 128: 243-247, 1973.

19. Wolff, S. M.: Biological effects of bacterial endotoxins in man. In Kass, E. H. and Wolff, S. M. (Eds.): Bacterial Lipopolysaccharides. Chemistry, Biology, and Clinical Significance of Endotoxins. Supplement, J. Infect. Dis., Vol. 128, 1973, pp. S259-S264.

20. Elin, R. J. and Wolff, S. M.: The role of iron in nonspecific resistance to infection induced by endotoxin. J. Immunol. 112: 737-745, 1974.

21. Wolff, S. M.: Familial Mediterranean fever (familial paroxysmal polyserositis). In Wintrobe, M. M., Thorn, G. W., Adams, R. D., Braunwald, E., Isselbacher, K. J., and Petersdorf, R. G. (Eds.): Harrison's Principles of Internal Medicine. Seventh Ed., New York, McGraw-Hill, Inc., 1974, pp. 1068-1071.

22. Wolff, S. M.: Midline granuloma. In Wintrobe, M. M., Thorn, G. W., Adams, R. D., Braunwald, E., Isselbacher, K. J., and Petersdorf, R. G. (Eds.): Harrison's Principles of Internal Medicine. Seventh Edition, New York, McGraw-Hill, Inc., 1974, pp. 1071-1072.

23. Gallin, J. I., Bujak, J. S., Patten, E., and Wolff, S. M.: Granulocyte function in the Chediak-Higashi syndrome of mice. Blood 43: 201-206, 1974.

24. Gallin, J. I. and Kirkpatrick, C. H.: Chemotactic activity in dialyzable transfer factor. Proc. Natl. Acad. Sci. USA 71: 498-502, 1974.

25. Gallin, J. I.: Radioassay of granulocyte chemotaxis. Studies of human granulocytes and chemotactic factor. In Sorkin, E. (Ed.): Chemotaxis, its Biology and Biochemistry. Basel, Karger. In press.

26. Gallin, J. I.: Abnormal chemotaxis: cellular and humoral components. In Dayton, D. H. and Bellanti, J. A. (Eds.): The Phagocytic Cell in Host Resistance. In press.

27. Gallin, J. I. and Rosenthal, A. S.: The regulatory role of divalent cations in human granulocyte chemotaxis: evidence for an association between calcium exchanges and microtubule assembly. J. Cell Biology In press.

28. Cohen, B. E. and Elin, R. J.: Vitamin A induced nonspecific resistance to infection. J. Infect. Dis. In press.

29. Wolff, S. M.: Pathogenesis of fever. In Sanford, J. P. (Ed.): The Science and Practice of Clinical Medicine. In press.

30. Dinarello, C. A., Goldin, N. P., and Wolff, S. M.: Demonstration and characterization of two distinct human leukocytic pyrogens. J. Exp. Med. In press.

31. Elin, R. J., Edelin, J. B., and Wolff, S. M.: Infection and immunoglobulin concentrations in Chediak-Higashi mice. Infect. Immun. In press.

32. Wolff, S. M.: Some challenges for future investigations in infectious diseases and clinical immunology. J. Infect. Dis. In press.

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Serial No. NIAID-18 (c)
1. LCI
2. Clinical Mycology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Immunology and Chemotherapy of Systemic Mycoses

Previous Serial Number: SAME

Principal Investigator: Dr. John E. Bennett

Other Investigators: Dr. James Balow
Dr. Michael Frank

Cooperating Units: Dr. David Alling, OSD, NIAID (No Project Number)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	3- 1/12			3- 1/12
Professional	1- 1/12			1- 1/12
Other	2			2

Objectives:

1) Examine humoral and cell mediated host response during cryptococcosis in man and in the experimental animal.

2) Improve the therapy of cryptococcosis.

Methods Employed:

1) Phagocytosis of a poorly encapsulated strain of Cryptococcus neoformans was measured by observation of stained smears taken at intervals during tumbling incubation at 37°C. Human neutrophils were obtained by Hypaque-Ficoll gradient followed by dextran sedimentation. C_3 was detected on cryptococci by agglutination or fluorescein antibody technique.

2) Mononuclear cell killing of cryptococci was studied in petri dishes rocked at 37°C for 24 hours. Human mononuclear cells were obtained by Hypaque-Ficoll gradient. Killing of cryptococci was measured by lysing the leukocytes in diluted Triton X-100, diluting aliquots in distilled water and plating in Sabouraud's agar.

3) Radiommunoassay for cryptococcal polysaccharide was performed using rabbit anticryptococcal antibody, ^{125}I labeled tyraminyl- or tyrosinylpolysaccharide and ammonium sulfate precipitation.

4) Guinea pig peripheral blood leukocytes were separated from blood by gelatin sedimentation of Hypaque-Ficoll gradient. Lymphocyte transformation was tested in microtiter wells using autologous serum or fetal calf serum. For MIF assay, peripheral blood lymphocytes were mixed with normal guinea pig peritoneal exudate cells, packed into 0.050 ml. capillary tubes and allowed to migrate in plastic wells.

Major Findings:

1) Studies have continued on mechanisms by which Cryptococcus neoformans may be opsonized for ingestion by human neutrophils. Coating the fungal cell with subagglutinating amounts of rabbit antibody did not opsonize the fungus. In collaboration with Dr. Michael Frank, it was attempted to add sequentially $C_{1,4,2,3}$ to antibody-coated cryptococci. Very little C_3 could be detected on the cryptococci, using either purified guinea pig or human $C_{1,4,2,3}$ and opsonization was negligible. When sheep erythrocytes were coated sequentially with chromium chloride, cryptococcal polysaccharide, anticryptococcal antibody and $C_{1,4,2,3}$, opsonization did occur. Cryptococci were pre-incubated with normal human serum or C_4-deficient guinea pig serum, washed well and tested for opsonins. Such cryptococci were heavily coated with C_3 and were readily phagocytized. These results indicate that the surface of cryptococci is a poor site for attachment of one or more of the early classical pathway components but the fungus affords a good surface for the attachment of early components in the alternate pathway.

2) A mixture of normal human peripheral blood monocytes and lymphocytes was found able to kill cryptococci if the fungal cells were coated with very small amounts of antibody. The antibody - dependent mechanism appeared to be nonphagocytic in that it occurred in the absence of enough serum to support phagocytosis and was not blocked by cytochalasin B, 10 µg/ml. This appears to be the first example of a microorganism being killed by this mechanism, previous studies by others having used erythrocytes or other tissue cells.

3) Studies continued on preparing radiolabeled cryptococcal polysaccharide of high specific activity. Biosynthesis using ^{14}C and ^{3}H-labeled precursors achieved specific activity of 10^{2} cpm/ µg. ^{125}I-tyraminylpolysaccharide yielded the same specific activity. Radioimmunoassay (RIA) for cryptococcal polysaccharide in human sera and cerebrospinal fluid was conducted, using ^{125}I-tyraminylpolysaccharide. Sensitivity and specificity of the assay were comparable to that obtained with the techniques now in use: complement fixation and latex agglutination. An important discrepancy was

that the apparent antigen concentration in sera from two of eleven cryptococcosis patients was much lower by RIA than by other tests. Both sera also contained anticryptococcal antibody upon Farr and indirect fluorescent antibody testing. The results suggested that the sera contained antigen-antibody complexes. In order to extend this observation and increase the sensitivity of the assay, ^{125}I-tyrosinylpolysaccharide of even higher specific activity has been prepared (10^6 cpm/ µg). With this reagent, RIA yielded a linear curve between 0.001 and 0.05 µg, or, using 0.1 ml samples, 0.01 and 0.5 µg/ml. Studies with this assay are now in progress.

4) A guinea model was developed for studying cellular events during sensitization or infection with Cryptococcus neoformans. Conditions were discovered for causing lethal or sublethal cryptococcosis and for best eliciting delayed skin reactivity, lymphocyte transformation and lymphokine production (MIF) with cryptococcal antigens. The MIF assay was developed in collaboration with Dr. Balow. Studies so far have found that sublethal cryptococcosis is associated with development of reactivity in all three tests. With lethal cryptococcosis, only preliminary data on skin reactions are available so far. Specific reactivity to cryptococcal antigens was found not to develop during such infections, though, if the animal had already been sensitized by dead cryptococci prior to infection, the reactivity did not wane during fatal cryptococcosis.

5) On February 5 and 6, an NIAID-sponsored meeting took place at N.I.H., in order to launch a collaborative study for the therapy of cryptococcal meningitis. Investigators from nine institutions, including this laboratory, derived a mutually satisfactory protocol for the comparison of amphotericin B therapy with a two drug combination: 5-fluorocytosine and low-dose amphotericin B. To date, six patients have been admitted to the study. This laboratory is contributing patients, performing drug sensitivity tests and serum drug bioassays, and, through the collaboration of Dr. David Alling, providing computer storage and retrieval of the data.

Significance to Biomedical Research and the Program of the Institute:

1) The peculiar inability of early classical but not alternate pathway complement components to bind to cryptococci suggests an important difference in the binding sites utilized by these pathways. A clearer understanding of these binding sites would be important in attempting to control complement-mediated reactions.

2) Antibody-dependent mononuclear cell killing of cryptococci is the first description of a mechanism by which antibody might assist host defense in cryptococcosis. Anti-cryptococcal antibody was found not to be opsonizing and not to lyse the cell in the presence of complement. The cell mediated killing offers an explanation as to why cryptococcosis patients with serum anticryptococcal antibody have a better treatment prognosis than seronegative patients. This study probably also is the first example of cell mediated, antibody-dependent, nonphagocytic killing of a microorganism.

3) The radioimmunoassay developed here is the most sensitive assay for cryptococcal antigen yet devised. It has the potential of improving serodiagnosis of cryptococcosis and permitting analysis of serum components such as antibody or complement that may complexed to circulating cryptococcal antigen.

4) Difficulty in producing lethal cryptococcosis in the guinea pig has prevented study of this valuable model. Now that a reproducible lethal infection can be induced and techniques have been developed for studying cell mediated events in single animals, it may be possible to produce and dissect the defects in cell function previously described by this laboratory in patients with cryptococcosis.

5) Conventional therapy of cryptococcal meningitis is associated with a 25% failure rate and permanent reduction of glomerular filtration rate by roughly one third. Combination therapy has the potential of both improving efficacy and reducing toxicity.

Proposed Course:

1) The defective binding of $C_{1,4,2,3}$ to antibody-coated cryptococci will be analyzed further with the collaboration of Dr. Frank, by measuring the number of molecules of each complement component that can be deposited on the cryptococcal surface. Covalent linkage of prosthetic groups or proteins to the cryptococcal surface may be tried in order to determine what characteristics can improve complement binding.

2) Attempts will be made to define the specific cell population responsible for antibody-mediated cytotoxicity. Early results suggest an adherent B-lymphocyte as the killer cell.

3) Sera and cerebrospinal fluid from patients with cryptococcosis will be tested for anticryptococcal antibody and cryptococcal antigen by the assay just devised. Antibody to alternate pathway components such as BGB will be utilized to test the hypothesis that circulating polysaccharide is complexed to such components. Sera suspected of containing antigen-antibody complexes will be subjected to conditions likely to dissociate such complexes and to separate physically the antigen from antibody. This should help confirm the presence of such complexes and also improve the sensitivity of the radioimmunoassay for antigen.

4) The guinea pig studies will be extended to examine lymphocyte transformation and, with the collaboration of Dr. Balow, MIF production by lymphocytes during fatal cryptococcosis.

5) The collaborative study of cryptococcal meningitis will continue until 100 patients have been studied.

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Honors and Awards:

Dr. John E. Bennett has engaged in the following activities outside the laboratory during the past year:

1) Appointed to the Ad Hoc Study Group on Bacterial and Mycotic Diseases, U.S. Army Medical Research and Development Command.

2) Reappointed to the Editorial Board of the journal "Antimicrobial Agents and Chemotherapy. In addition to reviewing 40 different manuscripts for this journal over the past 12 months, 13 manuscripts were reviewed for other journals.

3) Continues to serve on
   a. Advisory Committee to the Anti-Infective Agents Division, Bureau of Drugs, FDA.
   b. Editorial Board, "Control of Communicable Diseases in Man", American Public Health Association.

4) Invited lecturer at the following medical schools:

   Johns Hopkins, University of Pennsylvania (Philadelphia), Harvard, Pittsburgh, University of Connecticut, University of Maryland and 5 local hospitals. Invited participant in a symposium on immunity against mycoses, held at the national meeting of the American Society for Microbiology.

Publications:

1. Block, E.R., Jennings, A.E., and Bennett, J.E.: 5-fluorocytosine resistance in Cryptococcus neoformans. Antimicrob. Agents Chemother. 3: 649-656, 1973.

2. Levine, A.S., Siegel, S.E., Schreiber, A.D., Hauser, J., Preisler, H., Goldstein, I.M., Seidler, F., Simon, R., Perry, S., Bennett, J.E. and Henderson, E.S.: Protected environments and prophylactic antibiotics. A prospective controlled study of their utility in the therapy of acute leukemia. New Eng. J. Med. 288: 477-483, 1973.

3. Diamond, R.D., and Bennett, J.E. : Disseminated Nocardia brasiliensis infection. Arch. Intern. Med. 131: 735-736, 1973.

4. Diamond, R.D. and Bennett, J.E.: Disseminated cryptococcosis in man: decreased lymphocyte transformation in response to Cryptococcus neoformans. J. Infect. Dis. 127: 694-697, 1973.

5. Bennett, J.E.: Diagnoses and therapy of systemic mycoses in the immunosuppressed host. Transplant. Proc. 5: 1255-1257, 1973.

6. Block, E.R. and Bennett, J.E.: Stability of amphotericin B in infusion bottles. Antimicrob. Agents Chemother. 4: 648-649, 1973.

7. Diamond, R.D., May, JE., Kane, M., Frank, M.M. and Bennett, J.E.: The role of late complement components and the alternate complement pathway in experimental cryptococcosis. Proc. Soc. Exp. Biol. Med. 144: 312-315, 1973.

8. Block, E.R., Jennings, A.E. and Bennett, J.E.: Variables influencing susceptibility testing of Cryptococcus neoformans to 5-fluorocytosine. Antimicrob. Agents Chemother. 4: 392-395, 1973.

9. Bennett, J.E.: Chemotherapy of systemic mycoses. New Eng. J. Med. 290: 30-32, 320-323, 1974.

10. Diamond, R.D.: Antibody dependent killing of Cryptococcus neoformans by human peripheral blood mononuclear cells. Nature 247: 148-150, 1974.

11. Diamond, R.D., Bennett, J.E.: Prognostic factors in cryptococcal meningitis: A study of 111 cases. Ann. Intern. Med. 80: 176-181, 1974.

12. Block, E.R., Bennett, J.E., Livoti, L.G., Klein, W.J., MacGregor, R.R. Henderson, L.: The effect of hemodialysis on the plasma concentration and clearance of 5-fluorocytosine and amphotericin B in man. Ann. Intern. Med. In Press.

13. Diamond, R.D., May, J.E., Kane, M.A., Frank, M.D. and Bennett, J.E.: The role of the classical and alternate complement pathways in host defenses against Cryptococcus neoformans infection. J. Immunol. In press.

14. Young, R.D., Bennett, J.E., Geelhoed, G.W., Levine, A.S.: Fungemia in patients with compromised host resistance. Ann. Intern. Med. In press.

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Serial No. NIAID-23 (c)
1. LCI
2. Clinical Immunology Section
3. Bethesda, Maryland.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Observations on Secretory Immunoglobulins (Primarily Canine, but Includes Rabbits, Guinea Pigs and Humans).

Previous Serial Number: Same

Principal Investigator: Dr. Herbert Y. Reynolds

Other Investigators: Dr. Douglas M. Levin
Dr. Raphael Dolin

Cooperating Units: Dr. R. G. Mage, LI, NIH (Project No. NIAID 146)
Dr. D. L. Rosenstreich, LMI, NIDR, NIH (Project No:none)
Dr. N. F. Pierce, Dept. Medicine, Baltimore City Hospital, and Johns Hopkins University, Baltimore, Maryland
Dr. Myron J. Waxdal, LI, NIAID, NIH.(Project No. 149).

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	9/12			9/12
Professional	6/12			6/12
Other	3/12			3/12

Objectives:

1. To complete the molecular model for canine secretory IgA and identifying the assembly and interactions of secretory component (SC) and J-chain.

2. To elucidate the ultrastructure of secretory component and in vitro to study the susceptibility to proteolysis of dimeric serum and secretory IgA. Further, enzymes in gut epithelial cells (from in vitro cell cultures)

responsible for synthesis of SC and for the assembly of SC and IgA are being sought.

3. To examine the gut associated lymphoid tissue (Peyer's patches) in dogs and guinea pigs for its response to bacterial antigens and for its capacity to process antigens and to produce immunoglobulins (antibody).

4. Protective effect of secretory antibodies (IgA and IgG) in experimental gastrointestinal infections (cholera and non-bacterial infectious diarrhea).

Methods Employed:

Gel filtration and ion exchange chromatography, affinity and immunoabsorbant chromatography, standard techniques of zone electrophoresis, immunodiffusion; sedimentation velocity and equilibrium ultracentrifugation; equilibrium dialysis and fluorescence quenching for determination of binding energies; liquid scintillation spectroscopy; polyacrylamide disc gel electrophoresis; T iso-electrofocusing; amino acid analysis; and amino acid sequence analysis. Intestinal lymphoid cell tissue cultures, autoradiography; immunofluorescence and stimulation of lymphocytes by mitogens including PHA, Con A, endotoxin et cetera, measuring ^{3}H thymidine incorporation or lymphokine production (MIF).

Major Findings:

Secretory component (SC), predominantly attached to colostral and intestinal IgA through covalent (disulfide) bonding, has been dissociated, and isolated and its molecular weight of approximately 50,000 daltons established. Further isolation of J-chain, using polyacrylamide and isoelectric focusing separation techniques, from canine colostrum and serum IgM has been done. Further characterization of SC and J-chain with amino acid analysis and sequence are in progress. Likewise, intestinal IgA (Reynolds and Johnson, J. Immuno. 4: 888, 1970), is being prepared in batch quantities for use in proteolysis experiments and for use in antibody purification with Sepharose immunoadsorbents.

Peyer's patch lymphocytes have been isolated and successfully maintained in tissue culture from guinea pigs for periods exceeding 7 days. These experiments faced a formidable technical problem considering the bacterial contamination present on the gut surfaces. The lymphocytes have been identified as approximately 33-55% B-type and 40% T-type. Further, the functional capacity of Peyer's patch lymphocytes have been elucidated. T-cells in Peyer's patches are capable of mounting a graft vs. host reaction to allogeneic cells and of responding to similar cell surface antigens in two way mixed lymphocyte reactions; in addition, these Peyer's patch cells can be stimulated to liberate a lymphokine which induces monocyte chemotaxis. In summary, Peyer's patch cells have been shown to have efferent and afferent functions identified with cell mediated immunity and to behave in a manner similar to other peripheral lymphoid tissue.

Antibodies to cholera toxoid have been stimulated in serum and jejunal fluids of dogs immunized parenterally or locally in Thiry-Vella gut loops. Jejunal antibodies have been found to be both IgA and IgG and to possess antitoxin activity after intrajejunal immunization. Also, it was found that passively administrated IgG antitoxin antibody, purified from immunized

dogs, protected dogs from cholera infection by orogastric challenge or local challenge into Thiry-Vella jejunal loops. Thus, gastrointestinal infection or immunization results in both IgA and IgG jejunal antibody; when adequate levels of circulating humoral IgG antitoxin antibody exist in the immune host, additional resistance or protection against gastrointestinal infection occurs. It is likely that serum derived IgG antibody is important in protecting against gastrointestinal infection and works in concert with local secretory IgA antibody. Similar studies are being conducted in human volunteers infected with infectious agents which cause acute non-bacterial diarrhea.

Significance to Biomedical Research and the Program of the Institute:

These studies defining the molecular models of canine IgA's have made critical testing possible for the role of secretory piece in protecting secretory IgA from proteolysis and in a transport function. J-chain, likewise, is important in the assembly of IgA and probably IgM molecules. All these studies of canine IgA help to extend the homology of the mucosal secretory defense system in mammals and humans which is recognized as a major barrier for interaction with viral and bacterial micro-organisms by the host.

Studies on the function of gut lymphoid cells in birds and mammals, such as the Peyer's patch areas, are contradictory and may point to the tremendous species variations which will prevent easy generalities about function and significance. Therefore, each mammalian species will ultimately require study. The interaction of lymphoid cells which process antigens, such as gut bacterial antigens, and those which subsequently produce appropriate antibodies are likely to be vital in regulating the growth of normal intestinal flora and in the development of intestinal illnesses such as cholera and other diarrheal diseases. Now that these cells can be cultured in vitro and the various lymphocyte populations accurately identified, some of the confusion has been removed and hopefully more precise functions of these lymphoid aggregates can be found.

Proposed Course:

1. Further work on the structure of secretory piece and J-chain to show the interactions with IgA immunoglobulin and the elucidation of the chains' ultrastructure.
2. To elucidate the function of antigen processing cells in gut lymphoid tissue and to extend these observations to the bronchial associated lymphoid tissue.
3. To investigate the antibody response in humans following infection or immunization with cholera and non-bacterial agents causing diarrhea.

Honors and Awards: None

Publications:

1. Reynolds, H., Carta-Sorcini, M., and Mage, R.: The immunoglobulins derived from a V_H-C_H recombinant rabbit and its normal relatives. I. Measurements of heavy chain molecular weights and allotypes of colostral IgA. Immunochemistry. 10: 443-447, 1973.

2. Levin, D. M., Rosenstreich, D. L. and Reynolds, H. Y.: Immunologic responses in the gastrointestinal tract of the guinea pig. I. Characterization of Peyer's patch cells. J. Immunol.111 : 980-983, 1973.

3. Whicker, J. H., Neel, H. B. III, Kern, E. B., and Reynolds, H. Y.: A model for experimental vasomotor rhinits. Laryngoscope 83: 915-922, 1973.

4. Pierce, N. F. and Reynolds, H. Y.: Immunity to experimental cholera I. Secretory and humoral antitoxin response to local and systemic toxoid administration. J. Immunol. In press.

5. Pierce, N. F. and Reynolds, H. Y.: Immunity to experimental cholera II. Protective effect of humoral IgG antitoxin demonstrated by passive immunization. J. Immunol. In press.

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Serial No. NIAID-30 (c)
1. LCI
2. Clinical Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on the Interaction of Antibody and Complement in the Production of Immune Damage.

Previous Serial Number: SAME

Principal Investigator: Dr. Michael M. Frank

Other Investigators: Dr. Jeffrey Gelfand
Dr. John Atkinson
Dr. John Gallin
Dr. John Bennett
Miss Thelma Gaither
Mr. Robert Reinhart

Cooperating Units: Dr. E. Jaffe, NCI, NIH (Project No.: None)
Dr. Ira Green, LI, NIAID, NIH (Project No. 143)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	TOTAL
Total	1-9/12			1-9/12
Professional	1-1/12			1-1/12
Other	8/12			8/12

Project Description:

Objectives:

The role of antibody and of complement in the production of immune injury in vitro and in vivo will be explored. The factors which determine complement component levels in vivo will be defined and the factors which influence the rate of complement activation will be identified. An attempt will be made to develop new and better diagnostic methods for the better evaluation of patients with defects in host defense and patients with immunologic abnormalities.

Methods Employed:

Quantitative complement component assay, preparation of intermediates in the complement sequence, various complement components on the surface of cells, purification of complement components and antibodies to the various classes are routine in this laboratory. These methods employ the use of DEAE cellulose and C-M cellulose, Sephadex, electrophoretic techniques and ultra-centrifugation for the purification of immunoglobulins and complement component proteins. Cellular intermediates in the complement sequence (i.e. cells sensitized with antibody and reacted with various components of complement) are prepared by our own modification of standard methods and are used for titration of complement components. Efforts are made to determine the interrelationships and efficiency of various complement components reacting with other components from the same species and cross species lines.

In addition, complement cell intermediates have been built up on the surface of cryptococci as well as on the surface of erythrocytes coated with cryptococcal polysaccharide. Studies have in addition been conducted with animals with a genetically controlled C_4 deficiency. These animals have a complete block in their classical pathway but have an intact alternate pathway.

Major Findings:

Using serum of C_4 deficient quinea pigs, it has been possible to develop a new functional assay for human and guinea pig C_4 which is more sensitive than any previously developed method and which requires the availability of no purified components or complement-cell intermediates. This method should allow every hospital laboratory which has the capability of performing a whole complement titration (CH50) to test for C_4. The titer of C_4 in human serum by this method is in excess of 100,000 units/ml. The method has been used to perform a longitudinal study in 9 normal individuals. In 7/9 subjects C_4 titers were remarkably stable but in two there was variation over the 6 month study period which was unassociated with major clinical illness. This indicates that changes in C_4 levels in clinical specimens cannot be simply considered as proof of immunologic injury.

In extention of earlier studies it has been found that generation of chemotatic factors in human sera via activation of the classical or alternate complement pathway can be separated by inspection of the kinetic curves of chemotatic factor generation. Activation of the classical pathway leads to chemotatic factor generation within 10 minutes. Activation of the alternate pathway leads to a delay which may be as long as 15-20 minutes with some activating factors. Activation of both pathways produces the same chemotactic factor, C5a.

Earlier studies had suggested that corticosteroid therapy of guinea pigs leads to a progressive fall in the level of complement components. This has been examined carefully and it has been found, in metabolic turnover studies in the guinea pigs, that corticosteroid therapy leads to an increased rate of catabolism of the complement component C_3. Corticosteroid therapy does not lead to a decrease in C_3 synthesis.

Erythrocytes coated with purified antisheep erythrocyte antibodies and appropriate complement components have been used to study the nature of cellular infiltrates in tissue sections. Lymphocytes of the subclass which can differentiate into antibody forming cells have a prominent receptor for C_3. These lymphocytes may weakly bind erythrocytes coated with I_gG antibody. Monocytes in tissue section bind the I_gG coated erythrocytes avidly. The lymphocytes of thymic origin bind neither of these cell types in tissue section. Using these reagents it has been possible to identify these various cell types in normal and pathologic specimens.

Studies of the opsonization of cryptococci by the various pathways of complement activation have demonstrated that the classical pathway of activation requiring the pressure of the components C_1, C_4 and C_2 does not opsonize cryptococci. Properdin requires function of the pathway and the only function of the classical pathway is to make the pathway operate more rapidly. The biochemical basis for this interesting observation is being explored in detail.

Significance to Biomedical Research and the Program of the Institute.

These findings are significant at many levels. On the most practical level we have developed techniques to assess complement function which can be followed in laboratories which lack the tremendous sophistication usually required. We have developed new methods for identifying cells in tissue section and have shown their applicability to routine pathologic diagnosis. On the level of more basic studies, we have shown how the commonly employed drug cortisone may affect the complement system. We have studied the mechanism by which complement performs the essential function of opsonizing the pathogen Cryptococcus neoformans and this had led to more detailed studies of complement function.

Proposed Course:

These various leads will be systematically followed up to help determine

how complement functions in health and disease and how manipulation of the complement system may help in the diagnosis of human illness.

Honors and Awards: Dr. Frank was elected to membership in the American Society for Clinical Investigation.

Publications:

1. Sandberg, A. L., Snyderman, R., Frank, M. M., and Osler, A. G.: Production of chemotactic activity by guinea pig immunoglobulins following activation of the C3 complement shunt pathway.

2. May, J. E., and Frank, M. M.: Hemolysis of sheep erythrocytes in guinea pig serum deficient in the fourth component of complement. I. Antibody and serum requirements. J. Immun. III: 1661-1667, 1973.

3. May, J. E. and Frank, M. M.: Hemolysis of sheep erythrocytes in guinea pig serum deficient in the fourth component of complement II. Evidence for involvement of C^1 and components of the alternate complement pathway. J. Immunol. III:1668-1676, 1973.

4. Clark, R. A., Kimball, H. R., and Frank, M. M. : Serum chemotactic factors in an animal model of a complement deficiency state. In Braun, W and Ungar, J. (Eds.): "Non-Specific" Factors Influencing Host Resistance. Basel, Switzerland, S. Karger. pp. 205-214, 1973.

5. Frank, M. M., May, J. E. and Kane, M. A.: Contributions of the classical and alternate complement pathways to the biological effects of endotoxin. J. Infect. Dis. 128: S176-S181, 1973.

6. Frank, M.M., Ellman, L., Green, I., and Cochrane, C. G.: Site of deposition of C_3 in arthus reactions of C_4 deficient guinea pigs. J. Immunol. 110: 1447-1451, 1973.

7. Van Boxel, J. A., Paul, W. E., Frank, M. M. and Green, I.: Antibody-dependent lymphoid cell-mediated cytotoxicity: role of lymphocytes bearing a receptor for complement. J. Immunol. 110: 1027-1036, 1973.

8. Frank, M. M., Kane, M. A., and May, J. E.: Biological interactions of complement with endotoxin: Studies in C_4-deficient guinea pigs. In Braun, W and Ungar, J. (Eds.): "Non-Specific" Factors Influencing Host Resistance. Basel, S. Karger. pp. 323-331, 1973.

9. Clark, R. A., Frank, M. M., and Kimball, H. R.: Generation of chemotactic factors in guinea pig serum via activation of the classical and alternate complement pathways. Clin. Immunol. and Immunopathol. 1: 414-426, 1973.

10. May, J. E., Rosse, W., and Frank, M. M.: Paroxysmal nocturnal hemoglobinuria: alternate complement mediated lysis induced by magnesium. New Eng. J. Med. 289: 705-709, 1973.

11. Chused, T. M., Hardin, J. A., Frank, M. M. and Green, I: Identification of cells infiltrating the minor salivary glands in patients with Sjogrens syndrome. J. Immunol. 112: 641-648, 1974.

12. Van Boxel, J. A., Paul, W. E., Green, I. and Frank, M. M.: Antibody dependent lymphoid cell-mediated cytoxicity. J. Immunol. 112: 398-403, 1974.

13. Shevach, E., Edelson, R., Frank, M. M., Lutzner, M and Green, I.: A human leukemia cell with both band T cell surface receptors. Proc. Natl. Acad. Sci. USA.71: 863-866, 1974.

14. Jaffe, E. S., Shevach, E. M., Frank, M. M., Berard, C. W. and Green, I.: Nodular lymphoma: evidence for origin from follicular B lymphocytes. N. Eng. J. Med. 290: 813-819, 1974.

15. Diamond, R. D. and May, J. E.: The role of the classical and alternate complement pathway in host defense against Cryptococcus neoformans infections. J. Immunol. In press.

16. Jaffe, E. S., Shevach, E. M., Frank, M. D. and Green, I.: Leukemic reticuloeudotheliosis: presence of the histocyte I_gG receptor. Ann. Intern. Med. In press.

17. Gelfand, M. C., Elfenbein, G. J., Frank, M. D. and Paul, W. E.: Ontogeny of B lymphocytes, II. Relative rates of appearance of lymphocytes bearing surface immunoglobulins and complement receptors. J. Exp. Med. In press.

19. Gaither, T., Alling, D., and Frank, M. D.: A new one step method for the functional assay of the fourth component (C4) of human and guinea pig complement. J. Immunol. In press.

Serial No. NIAID-33(c)
1. LCI
2. Clinical Allergy and Hypersensitivity Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Pathogenesis of Delayed Hypersensitivity

Previous Serial Number: SAME

Principal Investigator: Dr. Charles H. Kirkpatrick

Other Investigators: Dr. Predrag Ugrinski
Dr. John Rhodes
Dr. Eric Ottesen
Dr. Peter Sohnle
Dr. John Gallin
Dr. Douglas Levin
Mr. Terrill Smith

Cooperating Units: Dr. David Rifkind, University of Arizona College of Medicine, Tucson, AZ
Dr. Nils Axelsen, University Copenhagen, Copenhagen, Denmark
Dr. Lauren Pachman, Children Memorial Hospital, Chicago, IL
Dr. Ira Green, LI, NIAID, NIH (no project no.)
Dr. Richard Edelson, Dermatology Branch, NCI (no project no.)
Dr. Ethan Shavach, LI, NIAID, NIH (no project no.)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	4-5/12			4-5/12
Professional	3-5/12			3-5/12
Other	1			1

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Project Description:

Objectives (Sub-project A):

1. Characterization of cellular immunity in patients with chronic mucocutaneous candidiasis.

Methods Employed:

Delayed hypersensitivity is assessed with intradermal tests with a variety of naturally encountered antigens.

T-lymphocytes are enumerated by the phenomena of spontaneous rosette formation with ovine erythrocytes. Peripheral blood lymphocytes from candidiasis patients and normal volunteers have been studied in short term cultures with antigens from C. albicans and phytohemagglutinin (PHA). The kinetics of dose-response relationships have been determined by labeling cells with ^{3}H-thymidine and counting incorporated radioactivity.

Production of the lymphokines, macrophage migration inhibition factor (MIF) and chemotactic factor (CTF) has been studied in cultured peripheral blood lymphocytes from patients with chronic mucocutaneous candidiasis. These assays involve exposure of lymphocytes to antigens in vitro, collection and concentration of the culture fluids and assays of MIF and CTF activities.

Major Findings:

Twenty-eight patients with chronic mucocutaneous candidiasis have been studied. Delayed skin responses have identified three immunological categories. Nine patients had normal delayed reactions to C. albicans as well as other antigens. Eleven patients had negative skin tests to C. albicans, but reacted to other antigens. Eight patients were negative to all antigens in the panel and anergy was confirmed by their failure to become sensitized to topical application of 1-chloro-2,4-dinitrobenzene. Three of these patients have immunological profiles similar to the Nezelof syndrome.

The experiments with cultured lymphocytes have disclosed further heterogeneity of the patients' immune responses. In most cases there was concordance between delayed skin reactivity and production of lymphokines. Only one skin test negative patient produced MIF while 2 skin test positive patients failed to produce MIF. Thus, there seems to be a close relationship between these phenomena.

There was also a direct relationship between lymphocyte transformation and positive skin tests, but it was less precise. Five skin test negative patients showed positive transformation. These data are suggestive that a subset of patients has antigen responsive cells but lacks lymphokine producing cells.

Significance to Biomedical Research and the Program of the Institute:

Data presented from this laboratory have shown that many patients with chronic mucocutaneous candidiasis have abnormalities of cellular immunity. Furthermore, the abnormalities are not the same from patient to patient. The observations presented above have been synthesized into a model for the pathogenesis of the disease which is described later.

Proposed Course:

These investigations will be continued in new patients referred for our study of chronic candidiasis in order to characterize their cell-mediated immunity.

Objectives (Sub-project B):

Development of programs for treatment of chronic mucocutaneous candidiasis which is based on clinical and immunological features.

Methods Employed:

In our series of patients, candidiasis either began between infancy and the third year of life, or between the ages of 8-13 years. Those patients with "late-onset" diseases were frequently under treatment with antibiotics for disorders such as acne vulgaris or furunculosis at the time of development of candidiasis.

We have proposed that patients with early onset disease have a defect in cellular immunity that has a congenital basis. In these patients, therapy must be directed at both eradication of the organism and restoration of intact cellular immunity. Patients with "late-onset" candidiasis may have a defect in host-defenses, but it has not been defined. From the results of the therapeutic trials, we believe that the immunologic abnormalities observed in this group of patients are acquired.

Amphotericin B has been used as the antifungal agent. The dosage range has been small, 800-1100 mgm, and administered over 7-8 weeks. When cultures from the nail folds become negative, the nails are avulsed in the belief that this is one of the reservoirs for recurrent infections.

Immunologic reconstitution has been done with transfer factor, a dialyzable substance obtained by disrupting leukocytes from skin test positive subjects. The optimal protocol for transfer factor is unknown, however, our protocol has been monthly injections for 4 months, then every fourth month. Recipients are monitored from in vivo skin responses and in vitro lymphocyte responses.

Major Findings:

The investigations conducted thus far have revealed additional defects in host defenses in patients with chronic candidiasis and have illustrated the importance of immunologic "staging" prior to implementation of a therapeutic program.

Five patients with "late-onset" candidiasis have received amphotericin B and nail avulsion and remained free of disease for 4, 3, 3, 2 and 1 years respectively. One patient relapsed in conjunction with iron deficiency and pregnancy. A second had a mild oral relapse. The other patients are doing well.

Five patients were treated with transfer factor alone. Although skin test reactivity and MIF production were observed in all five patients, there were no clinical responses.

Subsequently, two patients have been treated with the combined program of amphotericin B and transfer factor. Complete clearing of the mucocutaneous lesions occurred and both patients were in remission for several months. In one patient candidiasis of the tongue recurred after he was given erythromycin for a cold. This cleared with antifungal drugs but recurs periodically. The second patient was well for 9 months, but developed mucocutaneous recurrences when she began using an oral contraceptive. The lesion cleared with cessation of the pill and a short course of antifungal therapy. These patients are doing well 3 years after treatment.

One patient with a severe defect in cellular immunity received a thymus transplant. The procedure was followed by restoration of T-lymphocyte functions such as responses to phytohemagglutinin and concanavalin, E-rosette formation and acquisition of delayed-type skin responses. The reconstitutive effect persisted for eight months but then faded. However, the patient now responded to transfer factor.

Significance to Biomedical Research and the Program of the Institute:

From this experience it seems reasonable to assume that consideration of both clinical and immunological factors is essential for selection of effective therapy in candidiasis. Patients with "late-onset" disease may or may not have defective cellular immunity. The defect in these patients is apparently acquired and analogus to "immune-deviation" in experimental animals. Clearing of the fungal infection with amphotericin also reduces the tolerogenic load of antigen. In two cases the patients have become responsive to the antigens of candida, a event that would be expected in view of their obvious exposure to the antigen. The role of transfer factor in management of chronic infections and neoplastic diseases is unclear. No clinical trials with placebo or "negative" transfer factor have been done. An attempt to organize a cooperative trial of this type with NIAID support was not approved.

Proposed Course:

We plan to extend these treatment schedules to additional patients and continue the follow-up studies on the subjects who have been treated. As new treatment programs are developed they will be applied to the appropriate patients. An unresolved question with high priority is the importance of candida-specific transfer factor in treatment of chronic candidiasis.

Objectives (Sub-project C):

1. Characterization of the composition, properties and mechanisms of action of transfer factor.

2. Therapeutic trials with transfer factor in patients with defined immune deficiency syndromes.

Methods Employed:

The in vivo and in vitro tests of immunologic functions in man have been described in sub-project A. In addition, two major new endeavors have been added: characterization of a chemotactic agent in transfer factor and establishment of an animal model for transfer factor in the rhesus monkey.

Major Findings:

Reconstitution of immune functions in candidiasis as indicated by conversion of negative skin tests to positive has occurred in 5 recipients of transfer factor. Failures occurred in patients with Nezelof's syndrome. There has been good correlation between skin test reactivity and MIF production by cultured lymphocytes. In contrast, restoration of lymphocyte transformation responses has occurred only once. The immunologic basis of this disparity in candidiasis patients has not been established. It is of interest that similar findings have recently been reported in patients with the Wiskott-Aldrich syndrome. One possibility is that the lymphocytes which respond to antigens with thymidine incorporation and those which respond with lymphokine production comprise separate populations. If so, transfer factor preferentially affects the functions of the lymphokine producing cells. Alternatively, the differences may be due to differences in cell receptors which are dose dependent.

In collaboration with Dr. John Gallin, a chemotactic substance in transfer factor has been characterized. It is strongly chemotactic for polymorphonuclear leukocytes and weakly attractive for monocytes. It has a molecular weight of less than 5000 daltons and is not derived from complement. In brief, it apparently differs from previously described chemotactic factors. Preliminary studies have shown that the chemotactic activity and skin test-verting activity elute together from gel filtration columns.

A model for transfer factor activity has been established in rhesus monkeys. The transfers with transfer factor are specific but skin test sites must be biopsied for interpretation. The monkey model is currently being employed to monitor activity in column fractions and other experiments directed toward purification of transfer factor.

Previous studies with transfer factor have been criticized because naturally occurring antigens were employed. This raised the possibility that transfer factor did not confer new immunologic information onto the recipient, but had an enhancing or adjuvant-like activity that facilitated expression of previous sensitizations. Transfer factor from a donor with delayed allergy to a synthetic antigen has been studied in 3 monkeys and 2 patients. In no case was delayed allergy to the new antigen transferred.

Significance to Biomedical Research and the Program of the Institute:

These observations may be interpreted within the framework of the model for delayed hypersensitivity presented from this laboratory. The model envisions a multicellular system for antigen recognition and lymphokine synthesis analogous to that demonstrated for certain antibody responses in rodents. In the model, both antigen reactive cells and lymphokine producing cells would by "thymus-dependent".

The model therefore predicted that athymic patients with the Nezelof syndrome would not respond to transfer factor and this prediction has been substantiated in 3 cases.

The responses to transfer factor in the candidiasis patients may indicate that their immunological defect lies at a site that prevent expression of cellular immunity by lymphokine production. The lesion is apparently corrected or circumvented by transfer factor. Obviously, future experiments will be addressed to more precise delineation of the defect and the mechanism of action of transfer factor.

Proposed Course:

The experiments on purification and characterization of transfer factor will be continued.

Objectives (Sub-project D):

Search for linkage between chronic mucocutaneous candidiasis and histocompatibility antigens.

Methods Employed:

Peripheral blood lymphocytes have been typed for histocompatibility agents by the microcytotoxicity assay. This work was done through an agreement with Dr. Paul Terasaki, UCLA Medical School.

Major Findings:

The unusual distributions of HL-A antigens in patients with chronic mucocutaneous candidiasis are summarized in the table:

Antigen	Frequency (%)		
	Candidiasis Patients	Control	P
HL-A 3	39	18	<0.001
HL-A 7	39	14	0.01>P0.001
HL-A 12	39	11	<0.001
W 17	30	11	0.05

Significance to Biomedical Research and the Program of the Institute:

Studies in animals have shown that responsiveness to certain antigens is genetically controlled and linked to major histocompatibility antigens. Subsequently, human studies have shown unusual frequencies of HL-A antigens in patients with SLE, leukemia, Hodgkins disease and non-tropical sprue.

The finding of an unusual frequency of HL-A in patients with candidiasis may be evidence for linkage of immunologic unresponsiveness in these patients to a histocompatibility antigen. These studies will be pursued to clarify this point.

Proposed Course:

Although these studies will be extended to include new candidiasis patients as well as other patients with immune defects, the data on the 35 patients are currently being analyzed to see if the HL-A antigens identify a sub-group of patients with candidiasis.

Objectives (Sub-project E):

1. Characterization of serum antibody specificities in chronic mucocutaneous candidiasis.

2. Screening of patients sera for free candida antigens.

Method Employed:

Over 50 antigenic specificities have been identified in rabbit antisera to disrupted C. albicans. In collaboration with Dr. Nils Axelsen, the scientist who has identified these antigens, we have screened sera of candidiasis patients for their antibody profiles. The technique uses crossed antigen-antibody electrophoresis and gives qualitative and quantitative data.

By using patients serum in place of the candida fractions, the system has been adapted for detection of free antigens in serum.

Major Findings:

The serological profiles of six patients have been completed and six additional patients are under study. The qualitative and quantitative responses divided the patients into 2 groups. One group had antibodies against many antigenic fractions and the titers were generally very high. The second group had few responses and low titers. Of particular interest was the fact that the patients with numerous and generally high-titered responses were those with the most extensive and severe cutaneous disease. Free candida antigens have been identified on only one patient.

Significance to Biomedical Research and the Program of the Institute:

The mechanisms of the extensive cutaneous inflammation in candidiasis patients is unknown. The defective cellular immunity makes this route of inflammation unlikely. However, the marked serological responses in these patients may indicate a role for antigen-antibody-complement complexes in the pathogenesis of this form of candidiasis. This postulate is supported by the finding of free candida antigens in the serum from one patient. The alternative explanation for the serologic findings is that these responses are secondary to chronic intradermal immunization with candida.

Proposed Course:

The relationship of the antibodies to clinical manifestations of chronic candidiasis is now under study.

Objectives (Sub-project F):

1) Further characterization of immune responses to localized antigens.

Methods Employed:

Because the patients with chronic candidiasis are constantly exposed to antigen in a superficial site, a model of chronic, local antigen exposure was developed in the guinea pig. The antigen was a corneal graft and the study was conducted in collaboration with Dr. Predrag Ugrinski.

Major Findings:

Immunization with antigen in a superficial site induced systemic hypersensitivity to cornea and skin. Lymph nodes and peritoneal exudate cells were immunocompetent and could passively confer sensitivity to normal animals.

Significance to Biomedical Research and the Program of the Institute:

These observations demonstrate the systemic consequences of localized immunization. They are pertinent to the patients with candidiasis (a disease in which antigen is localized to the superficial layers of the skin), who lose their capacity to mount immunologic responses to candida. They may also apply to other disorders in which infections occur in apparently immunosuppressed patients (i.e., leprosy).

Proposed Course:

The principles of this model are now being directed to a candidiasis model in the guinea pig.

Objectives (Sub-project G):

1) Distributions of immunocompetent cells amongst tissue pools in disease.

Methods Employed:

In collaboration with Drs. Ira Green, Richard Edelson and Ethan Shevach, lymphocyte functions have been studied in vivo and in vitro in patients with the Sezary syndrome. The methods included skin testing, tissue biopsies and lymphocyte cultures.

Major Findings:

Even though the blood lymphocytes were poorly responsive to mitogens and antigens, they radily formed E-rosettes. In contrast, skin tests were often negative. The predominent cell in the skin was a T-lymphocyte.

Significance to Biomedical Research and the Program of the Institute:

These data indicate that T-lymphocytes may be sequestered in cutaneous compartments and may be deficient in the blood in certain diseases.

Proposed Course:

The application of these observations to chronic candidiasis and atopic dermatitis is under study.

Objectives (Sub-project H):

1) Definition of events affecting maturation of macrophages.

Methods Employed:

Macrophages possess a receptor for IgM and this receptor can be demonstrated with erythrocytes coated with IgM. The kinetics of receptor development are under study by Dr. John Rhodes.

Major Findings:

Fresh macrophages have a heterogenous distribution of receptors. However, oil-induced macrophages and cultured macrophages have high affinity receptors. Components of serum also influence the kinetics of receptor development.

Significance to Biomedical Research and the Program of the Institute:

Macrophage "activation" is an essential aspect of killing of microorganisms and tumor cells by these cells. Definition of factors that regulate macrophage activation may have application in "arming" cells and enhancing host defenses.

Proposed Course:

Studies of the affect of transfer factor on macrophage activation are planned.

Objectives (Sub-project H):

1) Relationship of oral immunization to immune responsiveness.

Methods Employed:

Guinea pigs were fed the contact allergen chlorodinitrobenzene and subsequently challenged by topical application of the chemical. The effects of cyclophosphamide on this phenomenon was studied. This work was conducted with Dr. Douglas Levin.

Major Findings:

Feeding animals marked reduced the incidence of contact allergy in guinea pigs and this phenomenon was transitory and animals became responsive after several weeks. This response was susceptible to cyclophosphamide.

Significance to Biomedical Research and the Program of the Institute:

Delayed hypersensitivity to candida is a common feature of chronic candidiasis and the oral cavity and gut provide a reservior for this organism in man. Although the guinea pig model is not a direct approach to this diseases, the analogy between oral exposure and suppress delayed allergy is clear.

Proposed Course:

To examine this model with C. albicans in the guinea pig.

Objectives (Sub-project I):

1) Interrelationship between cellular immunity, antibody synthesis and eosinophilia.

Methods Employed:

The model for this study was rats infected with Trichinella spiralis. Cellular immunity was studied with cells from mesenteric and popliteal nodes, blood and spleen stimulated in vitro with an extract of T. spiralis. Class specific antibody synthesis was measured by PCA and passive hemagglutination. Serial eosinophil counts were done. These experiments are being done by Dr. Eric Ottesen.

Major Findings:

Cellular immunity appears first in the regional (mesenteric) nodes after oral feeding of larvae. Subsequently cellular immunity appears in lymphocytes of the blood and spleen and later in remote (popliteal) nodes.

PCA antibodies and eosinophilia are found by the second week. There is a temporal variation of these responses which is very reproducible from animal to animal.

Significance to Biomedical Research and the Program of the Institute:

This study deals with the interface between immediate type hypersensitivity (homocytotropic antibody) and cell-mediated immunity. Both of these events are thymus-dependent. The data derived from the experiments will apply to the clinical events following certain parasitic infections, to atopic dermatitis and to the recently recognized syndrome of susceptibility to pyogenic infections and excessive serum IgE.

Proposed Course:

These studies will be continued and the possible contribution of the thymus in enhancing and suppressing the cellular responses will be studied by cell transfer.

Honors and Awards:

1. Extra-laboratory activities of the Principal Investigator -

   a) Chairman, NIAID Clinical Research Committee

   b) Project Officer, NIAID Contract for purification of antigen E, Ra3 and K from short ragweed pollen

   c) Member, Weather and Air Pollution Committee and Audiovisual Committee. American Academy of Allergy

   d) Chairman, International Committee, American Academy of Allergy

   e) Editorial Board, Journal of Cutaneous Pathology

Publications:

1. Abdou, N., Gaitt, R. A, Kirkpatrick, C. H., Leikin, S., Pachman, L. M., Rocklin, R. E., Spitler, L. and Wilcox, E.: Evaluation and therapy of patients with chronic mucocutaneous candidiasis. In Schwarz, M. R. (ed.): Proceedings of the Sixth Leukocyte Culture Conference. New York, Academic Press, 1972, pp. 841-845.

2. Kirkpatrick, C. H.: Steroid therapy of allergic diseases. Med. Clin. N. Am. 57: 1309-1320, 1973.

3. Kirkpatrick, C. H., Meek, J. C. and Rich, R. R.: Mechanism of allergy to components of commercial bovine thyrotropin. J. Allergy Clin. Immunol. 51: 296-302, 1973.

4. Axelsen, N. H. and Kirkpatrick, C. H.: Simultaneous characterization of free Candida antigens and Candida precipitins in patient's serum by means of crossed immunoelectrophoresis with intermediate gel. J. Immunol. Methods 2: 245-249, 1973.

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8. Ugrinski, P. S. and Kirkpatrick, C. H.: Corneal cellular immunity in the guinea pig. Am. J. Path. 74: 365-378, 1974.

9. Graw, R. G., Lohrmann, H.-P., Bull, M. I., Decter, J., Herzig, G. P., Bull, J. M., Leventhal, B. G., Yankee, R. A., Herzig, R. H., Kreuger, G. R. F., Bleyer, W. A., Buja, M. L., McGinnis, M. H., Alter, H. J., Whang-Peng, J., Gralnick, H. R., Kirkpatrick, C. H. and Henderson, E. S.: Bone marrow transplantation following combination chemotherapy immunosuppression (B.A.C.T.) in patients with acute leukemia. Transplantation Proc. In press.

10. Pachman, L. M., Kirkpatrick, C. H., Kaufman, D. H. and Rothberg, R. M.: The lack of effect of transfer factor in thymic dysplasia with immunoglobulin synthesis. J. Pediatrics. In press.

11. Kirkpatrick, C. H. and Gallin, J. I.: Treatment of infectious and neoplastic diseases with transfer factor. Oncology. In press.

12. Axelsen, N. H., Kirkpatrick, C. H. and Buckley, R. H.: Precipitins to Candida albicans in chronic mucocutaneous candidiasis studied by crossed immunoelectrophoresis with intermediate gel. Clin. Exp. Immunol. In press.

13. Edelson, R. L., Kirkpatrick, C. H., Shevach, E. M., Schein, P. S., Smith, R. W., Green, I. and Lutzner, M.: Preferential cutaneous infiltration by neoplastic thymus-derived lymphocytes: morphological and functional studies. Ann. Intern. Med. In press.

14. Levin, D. M., Lipsky, P. E. and Kirkpatrick, C. H.: Selective hypogammaglobulinemia with persistence of IgE, malabsorption and a nutritionally dependent, reversible defect in cell mediated immunity. Am. J. Med. In press.

15. Kirkpatrick, C. H.: Delayed (cellular) hypersensitivity. In Speer, F. and Dockhorn, R. J. and Shira, J. E. (eds.) Allergy and Immunology in Children. Springfield, IL, Charles C. Thomas, Chapter 10, pp. 130-143, 1973.

16. Kirkpatrick, C. H., Wells, S. A., Burdick, J. F. and Smith, T. K.: Effects of fetal thymus transplantation on defective cellular immunity. In Good, R. A. (ed.), Proceedings of the Second International Workshop on Primary Immunodeficiency Diseases in Man. New York, National Foundation. In press.

17. Kirkpatrick, C. H.: Restoration of cell-mediated immune responses with transfer factor. In Good, R. A. (ed.), Proceedings of the Second International Workshop on Primary Immunodeficiency Diseases in Man. New York, National Foundation. In press.

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18. Steinberg, A. D. and Kirkpatrick, C. H.: Concepts of immunologic intervention in the treatment of asthma. In Siegal, M. S. and Weiss, E. B. (eds.), Bronchial Asthma, Mechanisms and Therapeutics, Little, Brown and Co. In press.

19. Kirkpatrick, C. H. and Gallin, J. I.: The chemotactic activity of dialyzable transfer factor. II. Further characterization of the activity in vivo and in vitro. In Dayton, D. H. (ed.): The Phagocytic Cell in Host Resistance. In press.

Serial No. NIAID-36(c)
1. LCI
2. Biologic Structure Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Structural Correlates of Antigen and Mitogen Interaction with Immunocompetent Cells

Previous Serial Number: SAME

Principal Investigator: Dr. Alan S. Rosenthal

Project Description:

See the July 1, 1972 through June 30, 1973 project report. No work was done on this project during the past year. It is, therefore, terminated.

Serial No. NIAID-40(c)
1. LCI
2. Clinical Parasitology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Clinical Studies on Patients with Known or Suspected Parasitic Diseases

Previous Serial Number: SAME

Principal Investigators: Dr. Franklin A. Neva
Dr. Theodore E. Nash

Other Investigators: Dr. Eric Ottesen
Dr. Allen Kaplan

Cooperating Units: Dr. David W. Alling, OSD, NIAID, NIH (No project no.)
Dr. Richard Raizman, LPD, NIAID, NIH (Project no. 125-C)
Dr. Bjarne Bjorvatn, LPD, NIAID, NIH (No project no.)
Dr. David Wyler, LPD, NIAID, NIH (No project no.)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	1-4/12			1-4/12
Professional	1-4/12			1-4/12
Other				

Project Description:

Objectives:

1) Study pathophysiology of parasitic diseases.

2) Develop improved criteria to assess severity or extent of disease, indications for treatment, and reversibility of disease process.

3) Contribute to training of Clinical Associates, Guest Workers, and medical community in general concerning clinical diagnosis and management of parasitic diseases.

Methods Employed:

Patient referrals are made from private physicians and various agencies including the Peace Corps, Montgomery County Health Department and the NIH. Increasing numbers of patients are seen in the Outpatient facilities of the Clinical Center.

Quantitative egg counts are done on most patients with helminthic infections to better evaluate intensity of infection. Other appropriate diagnostic studies, as well as standard research procedures for parasitic diseases, are carried out in LPD. Lymphocytes are isolated from patients and exposed to mitogens and various parasite antigens for assessment of blast transformation with tritiated thymidine. Histamine release was measured fluorometrically from human basophils sensitized with serum antibody and challenged with parasite antigens.

Major Findings:

As in the past years a miscellany of patients with known or suspected parasitic infections have been seen with schistosomiasis probably constituting the largest number of inpatients. Another large category of cases has been giardiasis. Dr. Nash's observations on circulating antigen in schistosomiasis are reported separately (No. 121-K, LPD, NIAID) as are the complement studies in malaria (No. 134, LPD, NIAID).

A very resistant case of cutaneous leishmaniasis in a Peace Corps volunteer from Senegal, Africa was studied extensively. The parasite has been cultured and established in mice where it produces progressive facial and food-pad lesions. The mouse model with this interesting strain of L. tropica is being studied by a Guest Worker in LPD, Dr. Bjorvatn. The patient failed to respond to both pentavalent antimony and 1.2 grams of amphotericin even though his cell-mediated immune responses seemed adequate.

Esophageal motility studies were carried out on a Brazilian patient with chronic Chagas' disease manifested by mega-esophagus as well as by cardiomyopathy.

Several patients from Tonga (South Pacific) with microfilaremia were brought to the Clinical Center as a source of microfilarial antigens of W. bancrofti. Microfilariae were harvested by leukophoresis and purified to produce metabolic and somatic antigens. The antigens are being studied for their capacity to cause histamine release in human basophils sensitized by high-titered IgE serum from cases of eosinophilic lung. The point of these studies is to use possible specificity of the IgE response in these cases to determine filarial etiology of eosinophilic lung. Dr. Ottesen is also studying blast transformation in these cases before and after Hetrazan treatment.

Significance to Biomedical Research and the Program of the Institute:

The research and service activities of this Section complement the various infectious disease subspecialties within the LCI and help provide a well-balanced research and training program. Some of the patients seen have stimulated the initiation of LPD research projects and collaborative work between LPD and LCI.

Proposed Course:

Dr. Theodore Nash will be leaving for a clinical fellowship in June, 1974 and Dr. Wyler will take over for him to assist Dr. Neva. Dr. Raizman of LPD has also become increasingly involved in the care of patients with parasitic diseases. The mouse model with L. tropica will be maintained and studied further and it is hoped that collaborative work on allergic and immunologic aspects of filariasis can be continued and expanded to field studies.

Honors and Awards: NONE

Publications:

1. Neva, F. A. (Chief discussant): Acute adenopathy in a young man (Clinical Pathological Conference). In Gottlieb, L. S. and Southgate, M. T. (Coordinator and Editor). J. Am. Med. Assoc. 224: 1737-1746, 1973.

Serial No. NIAID-44 (c)
1. LCI
2. Clinical Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Pathophysiology of Autoimmune Hemolytic Anemia

Previous Serial Number: SAME

Principal Investigator: Dr. Michael M. Frank

Other Investigators: Dr. John Atkinson
Dr. Charles Jaffe
Dr. Herbert Reynolds

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	1-9/12			1-9/12
Professional	1-1/2			1-1/2
Other	8/12			8/12

Project Description:

Objectives:

To define the pathophysiologic function of antibody and complement on the surface of red cells in patients with acute hemolytic anemia and to determine the effect of these substances on red cell survival. To determine whether alterations in the state of activation of the cells of the fixed phagocytic system influences the clearance of sensitized erythrocytes. To determine in an animal model whether antimetabolites produce their clinical effect in treatment of Autoimmune hemolytic anemia (AIHA) by reducing clearance of sensitized cells. To extend these studies to human patients and to determine whether the findings obtained in guinea pigs can be

duplicated in humans.

Methods Employed:

Rabbits have been immunized with guinea pig erythrocytes utilizing a number of different routes of injection, and I_gM and I_gG immunoglobulin fractions of high purity have been prepared from the rabbit antisera. Fractions containing antibody with high avidity for guinea pig erythrocytes have been used to sensitize guinea pig erythrocytes. The number of complement fixing sites per erythrocyte formed by these antibodies has been determined by use of the Cla fixation and transfer test. The antibodies have been used to sensitize guinea pig erythrocytes labeled with 51 Cr and the half life of the cells studied after reinjection into the guinea pig.

In the most recent studies using this animal model guinea pigs were treated with daily injections of either azothiaprine or cytoxan for one week before study. Clearance studies were then performed. Also, in an extention of earlier studies complement deficient animals were treated with cortisone for 2 weeks before study in an attempt to explore the effect of cortisone on complement independent clearance.

Human anti A blood groups substance I_gM antibody was purified from human serum with high titer antibody by gel filtration and centrafugation. This antibody was utilized to sensitize human erythrocytes with a known amount of I_gM antibody, quantitated on a molecular basis by the Cla fixation and transfer test. Erythrocytes were removed from a normal donor, chromated with ^{51}Cr and then sensitized with I_gM antibody and reinfused into the donor.

Major Findings:

Neither azothiaprine nor cyclophosphamide decrease the clearance from the circulation of I_gM or I_gG sensitized erythrocytes. In some cases treatment with these drugs augments clearance. Low dose cytoxan has profound effects on the formed elements of the blood and marrow. Perhaps as a consequence of this low dose cyclophosphamide is the only situation we have studied in which therapy leads to a marked augmentation in the clearance of sensitized erythrocytes by the bone marrow rather than by the liver and spleen. The study suggests that the therapeutic efficacy of these drugs rests on their ability to inhibit antibody synthesis rather than on effects on RES clearance. Cortisone therapy was shown to markedly reduce the complement independent clearance of I_gM and I_gG sensitized erythrocytes. In fact, cortisone is more effective in reducing complement independent clearance than complement dependent clearance. The effect is dose dependent and there is little effect at very high levels of sensitization. The results help to explain the fact that cortisone has a greater therapeutic effect in those situations where antibody sensitized erythrocytes are destroyed by a complement independent mechanism.

The findings in human beings are as follows:

a) As few as 20 I_gM molecules/erythrocyte will lead to clearance (abnormal survival of the cells in the circulation). This number of antibody molecules cannot be detected by conventional methods of detection.

b) The site of removal of the cells is the liver. Sensitized erythrocytes are rapidly removed by the liver. A portion of the cells are ingested and a portion of the cells only adhere to Kupfer cells but are not ingested. These cells are released back into the circulation where they survive normally.

c) Clearance is complement dependent and the cells are not cleared in individuals with an intact alternate complement pathway but with a blocked classical pathway.

d) Clearance can be correlated with the number and type of C_3 fragment bound to the cell membrane after complement is activated. Each I_gM molecule leads to the deposition of 500-800 C_3 molecules on the erythrocyte membrane. The molecule form of the deposited C_3 is C_3b. These cells are immune adherence positive and are rapidly cleared from the circulation. Sensitized cells treated first with fresh serum and then with heated serum have a conversion product of C_3, C_3d on their surface. Such cells are immune adherence negative and survive normally in the circulation although they may be agglutinated by an anti C_3 Coombs reagent.

More recently in experiments performed in collaboration with Dr. Herbert Reynolds, we have attempted to identify the receptors on the human macrophage responsible for adherence of erythrocytes sensitized with antibody and various complement fragments. Thus far we have identified a receptor for the fragments of human C_3, C_3b and C_3d. We are attempting to learn why the C_3d receptor does not mediate clearance in vivo.

Significance to Biomedical Research and the Program of the Institute.

These studies define the role of antibody and complement in clearance of RBC's in patients with the disease, autoimmune hemolytic anemia. They represent the first molecular studies of the effect of immunoglobulin and complement on the survival of erythrocytes. They explain the finding, known for many years, that patients with hemolytic anemia may have Coombs positive cells in their circulation, which have a perfectly normal survival. Presumably, the cells have complement components placed on their surface by I_gM. The studies point out the in vivo importance of earlier studies from this laboratory, demonstrating that I_gM and I_gG handle complement components differently and produce different kinds and amounts of damage to cell surfaces. The studies provide a rational basis for understanding the mechanism of drug action in the treatment of these diseases. The most recent studies in humans provide evidence that the guinea pig constitutes a valid model for the study of these human illnesses and provides the basis for the first quantitative studies on the effect of I_gM antibody on erythrocyte survival in man.

Proposed Course:

The studies in man will be extended to provide data on each of the types of complement fixing and non complement fixing antibodies. The receptor studies will be continued in an attempt to find a valid explanation for the mechanism of cell clearance in these clinical illnesses.

Honors and Awards: None

Publications:

1. Atkinson, J. P. and Frank, M. M.: Effect of cortisone therapy on serum complement components. J. Immunol. III: 1061-1066, 1973.

2. Atkinson, J. P., Schreiber, A. D., and Frank, M. M.: Effects of corticosteroids and splenectomy on the immune clearance and destruction of erythrocytes. J. Clin. Invest. 52: 1509-1517, 1973.

3. Frank, M. M., Schreiber, A. B. and Atkinson, J. P.: In Proceedings of the Conference on "Phagocytic Cell in Host Resistance." Studies of the interaction of antibody complement and macrophages in the immune clearance of erythrocytes. In press.

4. Atkinson, J. P. and Frank, M. M. : Studies on the in vivo effects of antibody interaction of I_gM antibody and complement in the immune clearance and destruction of erythrocytes in man. J. Clin. Invest. In press.

5. Atkinson, J. P. and Frank, M. M.: The effect of bacillus calmette-guerin induced macrophage activation on the in vivo clearance of sensitized erythrocytes. J. Clin. Invest. In press.

Serial No. NIAID-45(c)
1. LCI
2. Biologic Structure Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Ultrastructural Correlates of the Cellular Events in Host Defense Mechanisms in Man and Experimental Animals

Previous Serial Number: SAME

Principal Investigator: Dr. Alan S. Rosenthal

Project Description:

See the July 1, 1972 through June 30, 1973 project report. No work was done on this project during the past year. It is, therefore, terminated.

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Serial No. NIAID-46(c)
1. LCI
2. Biologic Structure Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Immunobiologic and Ultrastructural Characterization of the Mechanism of Antigen Recognition

Previous Serial Number: SAME

Principal Investigator: Dr. Alan S. Rosenthal

Other Investigators: Dr. Jerrold J. Ellner
Dr. Dirk K. Greineder
Dr. Peter E. Lipsky
Mr. J. Thomas Blake
Mrs. Kerstin U. Cehrs

Cooperating Units: Dr. Ethan Shevach, LI, NIAID, NIH (Project No. 142)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	4-10/12	-	-	4-10/12
Professional:	3-10/12	-	-	3-10/12
Other:	1	-	-	1

Project Description:

Objectives:

1. To develop and apply techniques for the isolation of immunocompetent thymus-derived lymphocytes.

2. To characterize in a quantitative and qualitative fashion the response of such cells to a variety of *in vitro* correlates of cellular immunity such as antigen-dependent lymphocyte proliferation and macrophage inhibitory factor production.

3. To characterize the ultrastructure and physiology of these cells as contrasted with other types of lymphocytes.

4. To study the mechanism of lymphocyte recognition of and activation by antigen and mitogen using visible antigenic markers such as horseradish peroxidase (HRPO), HRPO-protein conjugates and radiolabelled antigens.

5. Characterize the role of macrophage-lymphocyte interaction in cell activation at a functional and ultrastructural level.

6. To study the genetic regulation of immune responsiveness and the role of surface membrane determinants in cell cooperation.

Methods Employed:

1. Determination of DNA synthesis of lymphocytic populations with and without addition of antigen by 4 hour pulse of ^{3}H-TdR incorporation. Results assayed by either/or both autoradiography or liquid scintillation spectrometry.

2. Preparation and isolation of partially purified population of lymphocytes from oil-induced peritoneal exudates or regional draining nodes subsequent (2-4 weeks) to immunization of inbred strain 2 and 13 guinea pigs with chemically defined antigens such as horse spleen ferritin, horseradish peroxidase or hapten protein conjugates.

3. Column purification: Peritoneal exudate cells (PEC) or lymph node cell suspensions were passed over glass bead columns and incubated at 37°C for one hour. Cells were then eluted with 10% guinea pig sera in minimal essential Eagle's media and the recovered cells studied.

4. Preparation of macrophage-rich oil-induced peritoneal exudate cells.

5. Ultrastructural analysis of antigen localization by means of autoradiography (isotopically labelled antigen) or cytochemistry (peroxidase coupled or direct use).

6. Binding of lymphocytes to a macrophage receptor can be assessed both in the presence and absence of antigen. Monolayers of guinea pig macrophages are prepared by adherence of monocyte-macrophages from oil-induced peritoneal exudates to Lab-Tek tissue culture chamber slides. After removal of non-adherent cells, 7×10^{6} lymphocytes are added to either control or antigen pulsed monolayers for varying periods of time, after which the non-macrophage bound lymphocytes are removed by washing and the monolayer fixed with 1% gluteraldehyde for light or electron microscopic analysis.

7. The uptake and fate in culture of soluble protein antigens is being studied functionally and by correlation of the state and localization of ^{125}I-labelled DNP-GPA. Additional studies with KLH, random copolymers of poly-L-glutamic acid-L-lysine and PPD are also being carried out.

8. Assay of the mixed leukocyte reaction (MLR) are carried out by assay of ^{3}H-TdR incorporation occurring when cells from a variety of strain 2 and 13 guinea pig lymphoid tissues are mixed and cultured for 5-7 days *in vitro*.

Major Findings:

1. Complex lymphoid cell mixtures placed over glass bead columns are

depleted of antibody forming cells and their precursors as measured by loss of plaque forming cells (PFC'S); immunoglobulin and antigen specific receptor bearing cells as measured autoradiographically. By contrast those cells which participate in in vitro correlates of cellular immunity such as antigen-dependent lymphocyte proliferation and elaboration of macrophage inhibitory factor (MIF) are excluded from the column.

2. There appears to be within the lymphoreticular system a segregation both functionally and anatomically of immune competence. Those cells which mediate cellular immune function appear to be sequestered within inflammatory sites such as oil-induced peritoneal exudates. These sequestered cells possess enhanced immune responsiveness producing greater inhibition of macrophage migration by a factor of 10 than an equivalent number of lymphocytes derived from the region nodes. Moreover, antigen recognition as indicated by new DNA synthesis in inflammatory exudate lymphocytes is 30-200 fold greater than an equivalent number of lymph node lymphocytes. The differences cannot be explained by presence or absence of macrophages, significant blastogenic factor release or desparities in the kinetics of cell responsiveness.

3. The peritoneal exudate lymphocyte have unique surface membrane changes on ultrastructural analysis.

4. The lymphocytes within the peritoneal exudate (PELS) appear to represent thymus-derived or T-lymphocytes. At least two types exist. One possesses a cell surface membrane modification, a uropod, whose formation is metabolically dependent. B lymphocytes which bear surface immunoglobulin do not appear to form uropods.

5. Activation of immunocompetent T lymphocytes is a complex phenomenon. While the exact mechanism is not established, our data strongly indicate a multi-step event in which an initial interaction of antigen with an immunologically naive macrophage occurs followed by T-cell macrophage interaction and thence antigen recognition. T lymphocytes do not themselves appear capable of direct interaction with soluble antigen. Regression analysis of cell density and studies employing metabolic inhibitors indicate a necessary physical interaction between the immunospecific T-lymphocyte and the macrophage being immunorelevant antigen.

6. The activation of antigen dependent T lymphocyte proliferation has been shown to require an obligatory presentation of antigen on macrophages rather than direct antigen interaction with the immunospecific T lymphocyte. Moreover studies with soluble protein antigens has demonstrated that histocompatibility linked membrane determinant play a rate limiting role in macrophage-lymphocyte cooperation.

7. Macrophages have been shown to possess surface membrane receptors from lymphocytes. Characterization of the kinetics, cellular specificity and ultrastructure of this phenomenon suggests that antigen independent binding of lymphocytes to macrophages plays an important role in antigen recognition by guinea pig T lymphocytes.

8. Addition of antigen to monolayers of macrophages in the presence of specifically immune lymphocytes results in a stabilization of otherwise reversible antigen independent binding of lymphocytes to macrophages. Such antigen dependent binding as assessed at 24 hours exhibits specificity and histocompatibility dependency and appears to represent an early morphologic correlate of antigen-mediated lymphocyte proliferation (Lipsky).

9. The uptake of soluble antigens by macrophages proceeds via a complex temperature and metabolic dependent process. At least two major pathways exist. The first involves binding of the protein to the surface membrane. Following surface binding, a metabolic dependent phase involves internalization of antigen (micropinocytosis). The fate of this non-degraded protein is not fully established. The second major pathway appears to involve macropinocytosis (fluid phase) probably representing at least 80% of the total uptake of protein. This later leads to lysosomal fusion and degradation to free aminoacid and small peptides. At present the micropinocytotic pathways appears to be immunologically relevant. Correlative functional and labelling studies indicates that immunogenecity of macrophage bound proteins decays with a half-life of approximately two days. While total intact protein content with time in culture remains constant, its accessibility to the lymphocytes is progressively diminished. Characterization and localization of this protein is currently under study (Ellner).

10. The nature of the stimulating and responding cells in the mixed leukocyte reaction (MLR) is not known. It is generally agreed that the likely responding cell is a T lymphocyte. Most controversial is the nature of the stimulating cell. In view of the initial and obligatory role of the macrophage in the recognition of soluble protein antigens by T lymphocytes and support of lymphocyte viability in vitro, we have examined the role of macrophages in the MLR. We have found that in the guinea pig there exists two pathways for initiation of the MLC. The major stimulating cell is the allogeneic macrophage. A bypass mechanism exists whereby responder macrophages either directly or indirectly facilitate recognition of gene products of the membranes of non-macrophages such as allogeneic T or B lymphocytes (Greineder).

Significance to Biomedical Research and the Program of the Institute:

The isolation and characterization of an enriched population of cellular immune effector cells should provide a useful system for study of the role of the cellular immune effector cells in immunologic surveillance and their contribution to the humoral limb of host defenses. Tumor immunity and transplantation immunity have been shown to rely to a great extent upon mechanisms of cellular immune function such as cytotoxicity and elaboration of other so-called "soluble mediators". A more fundamental understanding of the immunobiology of these crucial events and morphologic correlates may provide new insights into immune competence in man and information on the mechanisms of cellular regulation of immune responsiveness. Potential approaches to therapeutic manipulation of such events would be of immediate clinical relevance.

Proposed Course:

1. Characterize the mechanism of antigen recognition by T and B lymphocytes.

2. Characterize the role of the macrophage in in vivo and in vitro initiation of cell-mediated immunity in experimental animals.

3. Characterize the localization and fate of macrophage-associated antigen in in vitro antigen-mediated lymphocyte proliferation.

4. Explore the role of histocompatibility and genetic regulation in cell-cell interactions required for expression of cellular immunity.

Honors and Awards:

1. Chairmanship - Ninth Leukocyte Culture Conference, Williamsburg, Virginia, December 1-5, 1974.

Publications:

1. Stobo, J. and Rosenthal, A. S.: Biologically active concanavalin A complexes suitable for light and electron microscopy. Exp. Cell Res. 70: 443-446, 1972.

2. Waldron, J. A., Horn, R. G., and Rosenthal, A. S.: Antigen-induced proliferation of guinea pig lymphocytes in vitro: Obligatory role of macrophages in the recognition of antigen by immune T-lymphocytes. J. Immunol. 111: 58-64, 1973.

3. Rosenthal, A. S., Johnson, J. S., Rosenstreich, D. L., and Blake, J. T.: Evidence for a nonprecipitating antibody in the guinea pig. Int'l. Arch. Allergy Appl. Immunol. 44: 249-259, 1973.

4. Stobo, J. D., Rosenthal, A. S., and Paul, W. E.: Functional heterogeneity of murine lymphoid cells. V. Lymphocytes lacking detectable surface of immunoglobulin determinants. J. Exp. Med. 138: 71-88, 1973.

5. Cohen, B. E., Rosenthal, A. S., and Paul, W. E.: Antigen-macrophage interaction. I. Hapten-specific inhibition of antigen interaction with macrophages from immune animals. J. Immunol. 111: 811-819, 1973.

6. Cohen, B. E., Rosenthal, A. S., and Paul, W. E.: Antigen-macrophage interaction. II. Relative roles of cytophilic antibody and other membrane sites. J. Immunol. 111: 820-828, 1973.

7. Lipsky, P. E. and Rosenthal, A. S.: Macrophage-lymphocyte interaction. I. Characteristics of the antigen-independent-binding of guinea pig thymocytes and lymphocytes to syngeneic macrophages. J. Exp. Med. 138: 900-924, 1973.

8. Rosenthal, A. S. and Shevach, E. M.: Function of macrophages in antigen recognition by guinea pig T lymphocytes. I. Requirement for histocompatible macrophages and lymphocytes. J. Exp. Med. 138: 1194-1212, 1973.

9. Shevach, E. M. and Rosenthal, A. S.: Function of macrophages in antigen recognition by guinea pig T lymphocytes. II. Role of macrophages in the regulation of genetic control of the immune response. J. Exp. Med. 138: 1213-1229, 1973.

10. Rosenthal, A. S., Davie, J. M., Rosenstreich, D. L., and Cehrs, K. U.: Antibody-mediated internalization of B lymphocyte surface membrane immunoglobulin. Exp. Cell Res. 81: 317-329, 1973.

11. Waldron, J. A., Jr., Horn, R. G., and Rosenthal, A. S.: Antigen-induced proliferation of guinea pig lymphocytes in vitro: Functional aspects of antigen handling by macrophages. J. Immunol. 112: 746-755, 1974.

12. Rosenstreich, D. L. and Rosenthal, A. S.: Peritoneal exudate lymphocyte. III. Dissociation of antigen-reactive lymphocytes from antigen-binding cells in a T-lymphocyte enriched population in the guinea pig. J. Immunol. 112: 1085-1093, 1974.

13. Lipsky, P. E. and Rosenthal, A. S.: Antigen-independent physical interaction of guinea pig thymocytes and lymphocytes with macrophages. In Lindahl-Kiessling, K. and Osoba, D. (eds.): Lymphocyte Recognition and Effector Mechanisms. Uppsala, Academic Press, 1974, pp. 521-528.

14. Rosenthal, A. S. and Balow, J. E.: Mechanisms of glucocorticoid suppression of cell-mediated immunity. In Van Furth, R. (ed.): Mononuclear Phagocytes II. In press.

15. Rosenthal, A. S., Lipsky, P. E., and Shevach, E. M.: Morphological and functional correlates of macrophage-lymphocyte interaction. In Van Furth, R. (ed.): Mononuclear Phagocytes II. In press.

16. Rosenthal, A. S. and Rosenstreich, D. L.: The uropod: A specialized surface site for immunologic recognition. In Manson, L. (ed.): Biomembranes. New York, Plenum Press. In press.

17. Rosenthal, A. S., Lipsky, P. E., and Shevach, E. M.: Macrophage-lymphocyte interaction and antigen recognition. Fed. Proc. In press.

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Serial No. NIAID-47 (c)
1. LCI
2. Clinical Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973, through June 30, 1974

Project Title: Clinical Studies of Complement Abnormalities of Man

Previous Serial Number: SAME

Principal Investigator: Dr. Michael M. Frank

Other Investigators: Dr. Jeffrey Gelfand
Dr. John Atkinson
Dr. Franklin Neva
Miss Thelma Gaither

Cooperating Units: Dr. John Leddy, University of Rochester, Rochester, N.Y.
Dr. Wendell Rosse, Duke University School of Medicine, Durham, North Carolina

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	1-6/12			1-6/12
Professional	10/12			10/12
Other				

Project Description:

Objectives:

To define the role of complement in human illness and to develop better methods of therapy for immune damage mediated by complement.

Methods Employed:

Complement components are purified and used for estimation of component levels in human sera and spinal fluid. Double blind therapeutic studies are employed to test new methods of therapy in patients. Patients on experimental drug protocols are followed to determine signs of toxicity. New methods are devised to diagnose complement related diseases and to investigate their pathophysiology.

Major Findings:

During the past year the evaluation of the first patient with C_6 deficiency was completed. It was shown that this patient's serum possessed normal activity in chemotactic factor generation. It was unable to lyse bacteria, sensitized sheep erythrocytes or erythrocytes from patients with the disease paroxysmal nocturnal hemoglobinuria. The serum had normal opsonic function which underscores the importance of opsonic activity rather than bactericidal activity in protection from infection.

Evaluation has been completed of the tenth family with total C_2 deficiency. This is of interest because the propositus is the first individual to be identified with C_2 deficiency and dermatomyositis underscoring the interesting association between autoimmune disease and complement deficiency.

In collaboration with Dr. Frank Neva's group, we have explored the interrelations between malaria infection and complement activation. Humans with chronic infection with plasmodium vivax have a striking cyclycling of their complement levels which coincide with the parasite cycle. At the time of schizont rupture there is a profound fall in whole complement (CH_{50}) and in the level of the components C_1, C_4, and C_2. There is no major change in the levels of the later components, C_3 and beyond. Interestingly, this pattern of consumption resembles that noted in the disease hereditary angioedema except in that antigen antibody complexes appear to be responsible for the consumption and therefore C_1 is consumed. It is not known whether this pattern of C_1 activation participates in the production of clinical symptoms.

Study of patients with hereditary angioedema continues. We are continuing to follow a group of patients on long term treatment with epsilon amino caproic acid (EACA) in an attempt to further define the toxicity of this drug. A double blind study has been initiated to determine the effect of treatment with another agent, the progestational hormone Provera. Thus far four patients have participated in this double blind study using essentially the same protocal as that used in our earlier investigation of EACA. A standardized approach has been developed to many of the clinical problems which develop during the course of this disease in an attempt to reduce morbidity and mortality.

Significance to Biomedical Research and the Program of the Institute

These studies help define the role of complement in human illness and help to develop better means of therapy for sick individuals. The study of the rare patients with complement deficiencies has helped better define the importance of these components in disease states. Evaluation of drugs in therapy of hereditary angioedema is helping to treat these patients and moreover helping to identify those agents which may be useful in protecting against complement mediated disease.

Proposed Course:

These studies will be extended to evaluation of new therapeutic agents. The exploration of the role of complement in clinical illness will be continued.

Honors and Awards: None

Publications:

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2. Pearson, K. D., Buchignani, J. S., Shimkin, P. M., and Frank, M. M.: Hereditary angioneurotic edema of the gastrointestinal tract. Am. J. Roentgenol. Radium Therapy and Nuclear Med. CXVI: 256-261, 1972.

3. Green, I., Shevach, E.M., Stobo, J., Frank, M. and Heberman, R.: B cell, t cell and monocyte origin of the guinea pig L_2C leukemia, mouse lymphomas and human leukemias and lymphoblastic cell lines. In Jankovic, B. D. (ed.): Microenvinronmental Aspects of Immunity. New York, Plenam Publishing Company, 1972, pp. 491-498.

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5. Edelson, R. L., Smith, R. W., Frank, M. M., and Green, I.: Identification of subpopulations of mononuclear cells in cutaneous infiltrates. I. Differentiation between B cells, T cells and histiocytes. J. Invest. Dermatol. 61: 82-89, 1973.

6. Leddy, J. P., Frank, M. M., Gaither, T., Baum, J., and Klemperer, M. R.: Hereditary deficiency of the sixth component of complement in man. I. Immunochemical, biologic and family studies. 53: 544-553, 1974.

Serial No. NIAID-48 (c)
1. LCI
2. Clinical Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Antibody Responses in the Respiratory Tract Following Immunization with Pseudomonas aeruginosa Vaccine and Enhancement of Bacterial Phagocytosis and Killing by Respiratory Macrophages with Immune Opsonic Antibodies

Previous Serial Number: SAME

Principal Investigator: Dr. Herbert Y. Reynolds

Other Investigators: Dr. James E. Pennington
Dr. John A. Kazmierowski

Cooperating Units: Drs. Richard A. Robinson and Arthur S. Levine, Infectious Disease Section, Hematology and Supportive Care Branch, NCI, NIH (Project No. 6830).
Drs. Robert E. Wood and Paul A. DiSant-Agnese, Pediatric Metabolism Branch, NIAMD (Project No. 2c).
Dr. Harold H. Newball, Pulmonary Branch, NHLI, NIH (Project No.: None)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	1-4/12			1-4/12
Professional	1-1/12			1-1/12
Other	3/12			3/12

Project Description:

Objectives:

Pseudomonas aeruginosa is a serious opportunistic bacterial pathogen for patients with impaired host immunity due to hematological malignancies or from immunosuppressive therapy for various neoplastic or other systemic

diseases. Also, patients with extensive burns or structural lung diseases, particularly those who require assisted ventilation treatment, are at high risk to develop Pseudomonas infections. Pseudomonas colonization of the respiratory tract and subsequent pneumonia is particularly troublesome because the patient's response to antibiotics is often not dramatic and the microorganisms may not be eradicated. For example, pulmonary infections in children with cystic fibrosis are difficult to cure because antibiotics such as carbenicillin and gentamicin do not diffuse into bronchial secretions in sufficient concentrations to achieve inhibitory levels against the Pseudomonas organisms. Another approach to improve therapy of these pulmonary infections is that of immunological prophylaxis through the development of specific anti-Pseudomonas antibodies in serum and in the respiratory tract. These antibodies, particularly those with opsonic antibody activity, may help the lungs' cellular defense mechanism (pulmonary macrophages) by enhancing phagocytosis and killing of Pseudomonas.

The initial phase was conducted in experimental animals and has expanded now into a more general assessment of pulmonary host defenses which includes several human experimental protocols, employing a heptavalent Pseudomonas aeruginosa vaccine (Parke-Davis, Inc. BB-IND 185), and in the study of an immunosuppressed canine model of experimental pneumonia. In many ways the clinical projects are consolidated in this experimental pneumonia model (Pseudomonas aeruginosa, serotype II), done in collaboration with Dr. David C. Dale (LCI) and Dr. Robert Graw (NCI).

Methods Employed:

Rabbits are vaccinated with a variety of monovalent and polyvalent whole-cell or lipopolysaccharide Pseudomonas vaccines to observe the development of antibody in serum and bronchial secretions. Agglutinating antibodies, total titers and 2-mercaptoethanol resistant (IgG) titers, are measured. Isolation and purification of antibodies from serum and bronchial secretions employes gel filtration and ion exchange chromatography, sucrose density gradient ultracentrifugation, zone electrophoresis and other standard concentration and separation techniques. Macrophages, established in short-term cell monolayer cultures with well described techniques, are challenged with ^{14}C labeled bacteria, which have been previously opsonized; subsequent phagocytosis is measured with liquid scintillation spectroscopy and bacterial killing is assayed with standard agar pour plate cultures. Bronchial secretion proteins are quantitated by immunodiffusion precipitation methods. In addition, cellular immunity is evaluated by skin testing (histology) and in vitro culture of lymphocytes. Lymphocytes are stimulated to produce lymphokines (migration inhibition factor) and the migration of alveolar macrophages is studied.

Major Findings:

The type of specific anti-Pseudomonas antibody induced in the lungs or bronchial secretions depends upon the route of vaccination used - intramuscular (IM) or intranasal. Following parenteral (IM) immunization, IgG

agglutinative and opsonic antibody is present in lung secretions which diffuses into the lung from the blood; in contrast, intranasal immunization produces secretory IgA and secretory IgG antibodies in addition to a modest systemic or serum antibody response. These bronchial antibodies have been isolated and characterized. However, IgG seems to be the more important opsonic antibody in the lung on the basis of phagocytic and killing studies done with *in vitro* alveolar macrophages and ^{14}C labeled *Pseudomonas* organsims. Cell mediated immunity (CMI) develops transiently after immunization. Intranasal immunization results in immune respiratory lymphocytes which are capable of inhibiting the migration of normal alveolar macrophages for brief intervals (2-3 weeks) after primary immunization. In contrast, systemic(parenteral)immunization does not induce respiratory tract immunity.

Results of vaccination studies in three groups of susceptible patients to *Pseudomonas* infections (those with acute leukemia, cystic fibrosis, and structural lung diseases) have been completed and show that vaccination is tolerable in these patients, if small doses of steroids are combined in the syringe with the vaccine which effectively reduces systemic reactions to the lipopolysaccharide material. A good humoral antibody response can be obtained. A decrease in the number of Pseudomonas infections was not found in the vaccinated (n=22) *versus* control (n=20) groups of patients with acute leukemia because of the small number of available patients. Cystic fibrosis patients (n=12) responded well to the vaccine with high titers of agglutinating antibodies; immunization has not appeared to affect the infectious course or the colonization of their respiratory tracts with *Pseudomonas*.

Significance to Biomedical Research and the Program of the Institute:

Pseudomonas infections in patients with altered immunity or with structural lung disease is a major infectious disease problem which causes a high number of acquired infections in hospitalized patients. Recognizing that patient isolation is not feasible because of the ubiquity of the *Pseudomonas* organisms, which are part of the bacterial flora of many hospitalized patients, and that antibiotic therapy may be ineffective despite the availability of good anti-*Pseudomonas* antibiotics, immunization of high risk patients with *Pseudomonas* vaccines is a reasonable addition to existing therapy. A *Pseudomonas* polyvalent vaccine, developed by Parke, Davis and Co., Detroit, Michigan, is currently under investigation (BP-IND185)

As yet little attention has been given to the induction of protective antibodies in the respiratory tract and to the subsequent control of pulmonary colonization by *Pseudomonas*. Certainly, from the experimental animal studies described, it is possible to obtain specific bronchial antibody but to do so immunization must be prolonged enough to ensure development of IgG antibody. In the presence of the antibody which is available to opsonize *Pseudomonas* organisms, pulmonary macrophages *in vitro* are efficient in ingesting and killing these organisms. The role of cell mediated immunity, particularly in the lung, in activation of alveolar macrophages or enhancement of phagocytosis and intracellular killing must be determined.

Proposed Course:

1. Patients considered to be at high risk for Pseudomonas pulmonary infections, will be vaccinated with the Parke, Davis and Co., polyvalent Pseudomonas vaccine (Pseudogen) to determine the optimal vaccination regimen necessary to produce specific IgG bronchial antibodies. Observations concerning the protective value of these antibodies will be made. Intranasal or possible intrabronchial immunization will be done in patients with chronic lung diseases who are at increased risk for pseudomonas pneumonia in an attempt to maximize respiratory antibodies.

2. Continuation of the experimental pneumonia model in neutropenic and immunosuppressed dogs. Studies evaluating passive immunization with IgG agglutinating antibody will be done.

3. Studies with human respiratory macrophages and opsonic antibodies; similar to the animals experiments, will be continued. Further analysis of the cellular and protein milieu of the lower lung is underway. Patients and normal volunteers are undergoing bronchial lavage via transnasal fiberoptic bronchoscopy to obtain cells and protein components from the lower respiratory tract.

Honors and Awards: None .

Publications:

1. Reynolds, H. Y. and Thompson, R. E.: Pulmonary host defenses I. Analysis of protein and lipids in bronchial secretions and antibody responses after vaccination with Pseudomonas aeruginosa.J. Immunol. 111:358-368, 1973.

2. Reynolds, H. Y. and Thompson, R. E.: Pulmonary host defenses. II. Interaction of respiratory antibodies with Pseudomonas aeruginosa and alveolar macrophages. J. Immunol. 111: 369-380, 1973.

3. Pennington, J. E., Reynolds, H. Y., and Carbone, P. P.: Pseudomonas pneumonia: A retrospective study of thirty-six cases. Am. J. Med. 55:155-160, 1973.

4. Reynolds, H. Y., Thompson, R. E., and Devlin, H. B.: Development of cellular and humoral immunity in the respiratory tract of rabbits to Pseudomonas lipopolysaccharide. J. Clin. Invest. 53:1351-1359, 1974.

5. Reynolds, H. Y.: Pulmonary host defenses in rabbits following immunization with Pseudomonas antigens: The interaction of bacteria, antibodies, macrophages and lymphocytes. J. Infect. Dis. In press.

6. Dale, D. C., Reynolds, H. Y., Pennington, J. E., Elin, R. J., Pitts, T. W. and Graw, R. G.: Experimental Pseudomonas pneumonia: Controlled trial of granulocyte transfusion therapy. J. Clin. Invest. In press.

7. Reynolds, H. Y. and Newball, H. H.: Analysis of proteins, lipids and respiratory cells obtained from human lungs by bronchial lavage. J. Lab. Clin. Med. In press

8. Pennington, J. E., Reynolds, H. Y., Wood, R. E., Robinson, R. A. and Levine, A. S.: Use of a Pseudomonas aeruginosa vaccine in patients with acute leukemia and cystic fibrosis. Am. J. Med. In press.

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Serial No. NIAID-51 (c)
1. LCI
2. Clinical Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Human Responses to Salmonella typhosa Vi Antigen (Clinical Research #73-I-01)

Previous Serial Number: Same

Principal Investigator: Dr. Herbert Y. Reynolds

Other Investigators: Dr. Douglas M. Levin

Cooperating Units: Dr. K. H. Wong, Division of Bacterial Products, BB, FDA, Rockville, Maryland

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	1			1
Professional	9/12			9/12
Other	3/12			3/12

Project Description:

To test safety and immunogenicity of Salmonella typhosa Vi antigen in human volunteers. Typhoid vaccines in present use are quite reactive, producing local and systemic toxicity after injection, and do not provide complete protection against the disease. Typhoid vaccine trials comparing an acetone-inactivated dried vaccine with a heat- and phenol-killed vaccine demonstrated better protection with the acetone-treated preparation. This has been attributed to the destruction of Vi antigen by the heat-phenol process. Vi antigen prepared from citrobacter organisms shares antigenic determinants with S. typhosa Vi. Such a citrobacter Vi was used in human challenge studies. Protection was less than that seen with whole cell vaccine, but some protection occurred. For this reason, a Vi preparation

from S. typhosa has been prepared ($Vi\text{-}Ty_2$) by a mild precipitation procedure. This preparation contains 3.5% endotoxin, and at a 50 µg human dose is 23-fold less toxic than a human dose of conventional vaccine, as tested in the actinomycin-D mouse endotoxin assay model. Protective potency in the mouse challenge model is approximately 16 times that of conventional vaccine, comparing them at doses relative to a human dose, and 176 times more potent than the Vi preparation used in the human challenge studies. In addition, the $Vi\text{-}Ty_2$ is approximately three times more active in stimulating the in vitro thymidine uptake by mouse spleen lymphocytes than a citrobacter Vi preparation or than the conventional vaccine. Using the standard assays prescribed by Federal Regulations for licensed products, it is safe and non-toxic in guinea pigs and mice in toxicity assays, and is sterile.

Human studies to determine reactivity and immunogenicity in comparison with an acetone-killed and dried conventional typhoid vaccine (lot 6A, Bureau of Biologics reference standard) are underway. Data relative to the $Vi\text{-}Ty_2$ preparation was filed with the Bureau of Biologics for IND review prior to initiation of human studies. The vaccine preparation from which the 6A lots were derived has been used safely in humans in the past, and the 6A lots have passed standard safety and sterility tests.

Methods Employed:

Healthy normal subjects without history of previous typhoid immunization will be utilized. Blood samples (250-300 ml total for entire protocol will be obtained for assay of antibody and cellular immunity prior to and at intervals following immunization over a 6-week period, and, where possible, 3-6 months later. Antigen will be injected intramuscularly following the drawing of a blood sample at 0 and 4 weeks. Observation of the volunteer will be carried out after each injection. This will include recording of vital signs four times daily for at least 3 days, and questioning of the patient for symptoms of systemic toxicity, including fatigue, anorexia, malaise, headache, and myalgia for at least 3 days.

Volunteers will be alternatively assigned to two groups. Five volunteers in the first group will receive 25 µg of $Vi\text{-}Ty_2$ in phospate buffered saline. Five volunteers in the second group will receive 0.5 ml of conventional acetone typhoid vaccine (type 6A Bureau of Biologics reference standard vaccine). At least one week later, a second five volunteers in the first group will receive 50 µg of $Vi\text{-}Ty_2$, and at least one week later a third group of five volunteers will receive 100 µg. A booster of their original dose will be given to all volunteers at 1 month if no untoward reactively is noted.

All blood specimens will be analyzed for bactericidal and hemagglutinating antibody titers to S. typhosa. Lymphocytes will be separated from selected specimens and examined by MIF and thymidine uptake techniques for reactivity to typhoid antigens.

In addition to these studies, salivary and parotid secretions (Kirby cup) will be collected from selected volunteers at various times during the

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course of the study, and examined for secretory antibody. Also, intradermal injections of no more than three different dilutions of the Vi antigen (1 µg or less) or the whole cell vaccine will be performed 2 days prior to the initial immunization, and one week following the second injection, to assess early and delayed skin hypersensitivity. A skin test of the appropriate diluent will be done concomitantly, for comparative purposes.

Major Findings:

Phase I studies have been completed and included the primary immunization of three groups of normal volunteers (groups of 5) with various doses of the Vi-Ty_2 vaccine: 25 µg, 50 µg and 100 µg. Booster injections were also given. In addition, controls were immunized with the conventional 6A typhoid vaccine. It was found that the 50 µg dose of Vi-Ty_2 gave good hemagglutinating antibody titers yet produced minimal, and very acceptable and tolerable, local side reactions at the injection site. Convincing evidence of developing cellular immunity, assessed by skin test reactivity and peripheral blood lymphocyte lymphokine release (migration inhibition factor effect on guinea pig peritoneal macrophage mobility) was not found. To confirm the effect and response of the 50 µg Vi-Ty_2 dose, ten additional volunteers were immunized with this dose and good antibody titers were recorded. The data from these studies is being compiled.

Significance to Biomedical Research and the Program of the Institute:

Hopefully, an improved vaccine to protect against typhoid fever will be found.

As the Vi-Ty_2 preparation is substantially reduced in toxicity from conventional typhoid vaccine, markedly reduced local and systemic reactions can be expected. Animal work would indicate that this new vaccine has good immunogenicity so that antibody response and possibly cellular immunity will be good.

Proposed Course

1. Further purification of the Vi-Ty_2 antigen is being done to reduce the side reactivity of the vaccine (by better removal of endotoxin) yet hopefully not to impair the vaccine's antigenicity. Further volunteer immunization with this new Vi-Ty_2 preparation will be undertaken.
2. Field trials with the vaccine may be undertaken.

Honors and Awards: None

Publications: None

Serial No. NIAID-52 (c)
1. LCI
2. Clinical Immunology Section
3. Bethesda, Maryland

PHS-NIH

Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Antibiotic Kinetics in Respiratory Secretions

Previous Serial Number: Same

Principal Investigator: Dr. Herbert Y. Reynolds

Other Investigators: Dr. James E. Pennington

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	1			1
Professional	9/12			9/12
Other	3/12			3/12

Project Description:

Objectives:

A variety of gram negative bacteria are pathogens for patients with impaired host immunity due to hematological malignancies or immunosuppressive therapy for various neoplastic or other systemic diseases. Pseudomonas aeruginosa colonization of the respiratory tract and subsequent pneumonia is particularly troublesome because the patient's response to antibiotics often is not dramatic and the microorganisms may not be eradicated. For example, pulmonary infections in children with cystic fibrosis are difficult to cure because antibiotics such as carbenicillin and gentamicin do not diffuse into bronchial secretions in sufficient concentrations to achieve inhibitory levels against the Pseudomonas organisms (Marks et al, J. Pediat. 74: 822, 1971).

Therefore, within the scope of a larger project to investigate pulmonary host defenses (Serial #NIAID-48(c)), we were interested to study the penetration of antibiotics into the lung and respiratory secretions and to observe the clearance of antibiotics from these secretions. An animal model was designed to study the kinetics of antibiotics in normal respiratory secretions. These studies are to be extended to patients where possible. Initially, antibiotics used specifically for Psuedomonas infections were evaluated.

Methods Employed:

Anesthetized and intubated dogs are used; blood (serum) and bronchial secretion samples are simultaneously obtained following administration of a variety of antibiotics which has included: penicillin G, ampicillin, getamicin, carbenicillin, tobramycin and 5-fluorocytosine. Antibiotic concentrations are measured using a cup plate agar method (Bennett et al, Applied Microbiol. 14: 170, 1966). Bronchial secretions are also analyzed for protein content and quanitatively for albumin and various classes of immunoglobulins (Reynolds and Johnson, J. Immunol. 105: 698, 1970).

Major Findings:

Following injection of maximal doses of gentamicin or carbenicillin, levels in normal bronchial secretions were obtained that would inhibit about 63% and 43%, respectively, of 1500 Pseudomonas aeruginosa isolates for which the mean inhibitory concentrations (MIC) were known. Tobramycin concentrations in bronchial fluids were equal to the MIC's reported for about 70% of Pseudomonas isolates. In contrast, penicillin G, which has demonstrated efficacy for such lung infections as pneumococcal pneumonia, was found to achieve levels 40 X the MIC's of over 99% of pneumococci isolated at NIH. The particulars of the canine model were also detailed in hopes that this might become an acceptable in vivo model for studying respiratory antibiotic clearances. Of note is that clearance of antibiotics from the lungs is appreciably slower than from serum which raises possibilities for increasing the effective concentration of antibiotics in the respiratory tract.

Significance to Biomedical Research and the Program of the Institute:

Antibiotic treatment for certain kinds of bacterial pneumonia produces variable rates of cure and often seems to be ineffective in specific infections such as Pseudomonas pneumonia where the mortality rate in most clinical series approaches 80%. A factor of importance is that antibiotics as currently administered may not reach sufficient concentrations in the lung or bronchial secretions to inhibit growth of the infecting microorganisms. Methods to increase the effective level of antibiotics in the bronchial tract, such as direct instillation by aerosol or combinations of parenteral and aerosol administration, should be investigated.

Information about the clearance of antibiotics from the respiratory secretions is sparse compared with similar data about renal or hepatic clearance and excretion. The dog model used in this project may be useful for these studies.

Proposed Course:

1. Additional antibiotics will be examined in the dog model such as those used in the treatment of chronic bronchitis (Ampicillin, tetracycline and trimethoprin and sulfa) and fungal disease (5-FC).
2. Anti*Pseudomonas* antibiotics will be measured in respiratory secretions of immunosuppressed dogs with experimentally induced *Pseudomonas* pneumonia.
3. Confirmation of antibiotic clearance in respiratory secretions will be attempted in suitable patients with tracheostomies.

Honors and Awards: None

Publications:

1. Pennington, J. E. and Reynolds, H. Y.: Concentrations of gentamicin and carbenicillin in bronchial secretions. *J. Infect. Dis.*128:63-68, 1973.

2. Pennington, J.E. and Reynolds, H. Y.: Tobramycin in bronchial secretions. *Antimicrob. Agents Chemother.* 4:299-301, 1973.

Serial No. NIAID-53(c)
1. LCI
2. Medical Virology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Etiology and Clinical Characteristics of Acute Infectious Nonbacterial Gastroenteritis

Previous Serial Number: SAME

Principal Investigator: Dr. Raphael Dolin

Other Investigators: Dr. Millicent Coker
Mr. Holly A. Smith

Cooperating Units: Dr. Albert Z. Kapikian, LID, NIAID, NIH (Project No. 66H)
Dr. Frederick Klipstein, University of Rochester Medical School, Rochester, New York
Dr. Arnold S. Levy, Laboratory of Digestive and Hereditary Diseases, NIAMD, NIH (No project no.)
Dr. Thomas S. Thornhill, LID, NIAID, NIH (Project No. 66H)
Dr. Richard G. Wyatt, LID, NIAID, NIH (Project No. 66H)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	1 - 6/12			1 - 6/12
Professional	10/12			10/12
Other	8/12			8/12

Project Description:

Objectives:

1) To define the biologic, immunologic and epidemiologic properties of the etiologic agents (viruses) of acute infectious nonbacterial gastroenteritis.

2) To develop in vitro culture systems and/or laboratory animal models with which to study these agents.

3) To investigate the pathogenesis of and host response to infection with these agents in man, by the experimental induction of disease in normal volunteers.

4) To examine the possible role of these agents in chronic gastrointestinal diseases of unknown etiology.

5) To devise control measures based on the above information.

Methods Employed:

1) Source of Viral Agents. Seven viral agents have been obtained from naturally occurring outbreaks of acute infectious nonbacterial gastroenteritis (AINBG). These agents, present in diarrheal stools of acutely ill cases, are maintained as infectious stool filtrate suspensions, which can be examined in the laboratory or can be employed to infect susceptible volunteers.

2) In Vitro culture systems: The above viruses have not been detected in standard tissue culture systems despite extensive searches, employing direct and indirect methods of virus identification. Replication of these viruses has been demonstrated in a recently developed system, human fetal intestinal organ cultures, by infecting volunteers with blind passaged material. Therefore, particular emphasis is being placed on *in vitro* detection of these agents in organ culture, employing light, fluorescence and electron microscopy, viral interference, metabolic alterations, and enzyme production.

3) Volunteer studies in man. After rigorous safety tests, normal volunteers are infected via oral administration of these viruses. The inocula undergo certain physical treatments (e.g., ether treatment, isopycnic centrifugation) to define biophysical properties of the infectious agents. In addition, the host response is studied by obtaining blood and stool samples, respiratory washings, per-oral jejunal biopsies and secretions, sigmoidoscopic examinations and rectal biopsies. Peripheral blood lymphocyte cultures are established to investigate the role of cell mediated immunity in disease. Biopsy specimens, as well as organ cultures established from those specimens, are examined for ultrastructural changes, immunoglobulin production, and enzyme synthesis.

4) Immune electron microscopy: Stool filtrates or other virus containing fluids are reacted with convalescent sera containing antibodies to the virus. The virus antibody complex is centrifuged onto a grid and examined by negative staining under the electron microscope. The aggregated virus can thus be identified and its properties further defined. Conversely, employing filtrates with known amounts of virus, antibodies can be detected in serum and secretions.

Major Findings:

1) Antigenic relationships among the viruses of AINBG: viral agents from two distinct outbreaks of AINBG (Honolulu - "H" agent, and Montgomery, County - "MC" agent) were compared with the Norwalk agent for antigenic relatedness. Three of six volunteers previously ill with Norwalk disease, demonstrated illness after infection with H virus; while none of 8 volunteers previously ill with Norwalk disease manifested illness after inoculation with the MC virus. Following infection with the Hawaii virus, 2 of 3 volunteers became ill with Norwalk virus, and none of 4 became ill with Montgomery virus. These studies suggested that Norwalk and Hawaii agents were antigenically dissimilar, while Norwalk and MC agents appear to be related. Further studies are planned to investigate the relationship of H to MC virus.

Assessment of serum antibody by immune electron microscopy showed cross reactions in Norwalk and MC disease, but no cross reaction in between Norwalk and H convalescent sera.

2) Intestinal Histopathology: Since July 15, 1973, per oral jejunal biopsies were obtained on 12 volunteers, before, 72 hrs after, and two weeks following oral administration of Norwalk (4) or Hawaii agent (8). Light and electron microscopic histopathology was limited to those volunteers who became ill (7). With the Hawaii virus, the mucosa remained intact, with blunted villi and mild mononuclear infiltration in the lamina propria. Epithelial cells showed mild vacuolization, but had markedly shortened and distorted microvilli. Thus, the histopathological changes were similar but not identical to that described previously for the Norwalk agent (Agus, Dolin). This suggests that this basic lesion may be a general feature of infection with viruses of AINBG, with some limited strain or type variation.

Biopsies were also examined for fluoride stimulatable adenyl cyclase activity (Levy, Klaevemann). However, only one of seven ill volunteers showed a significant change in activity during illness. Human fetal intestinal organ cultures infected with Norwalk and Hawaii agents also failed to show consistent adenyl cyclase changes.

Normal sigmoidoscopies and rectal biopsies, along with the absence of fecal leukocytes in Norwalk and Hawaii-induced disease suggested that intestinal infection with these agents was limited to the small intestine and spared the colon.

3) Humoral, secretory and cell mediated immunity to Norwalk agent and Hawaii agents. Employing immune electron microscopic (IEM) aggregation as an assay, less than 10% of normal volunteers examined had 0-2+ detectable serum antibody, while greater than 90% had conversion to 3+ or greater after illness. Since the frequency of illness in sero "negative" volunteers was approximately 50%, other factors in host resistance to infection were investigated. The role of local antibody was begun to be studied by column and sucrose gradient separation of immunoglobulin from intestinal secretions,

stool extracts, and intestinal biopsy organ culture explants. These immunoglobulins will be tested in IEM for antibody activity.

Cell mediated immunity was investigated by thymidine incorporation of PHA stimulated peripheral lymphocyte cultures from volunteers prior to,during, and 5-14 days after Norwalk and Hawaii induced illness. 6 of 9 volunteers demonstrated a 2.5-13 fold suppression of PHA response during acute illness (mean 4.7) while 8 of 9 volunteers who remained well following inoculation demonstrated 1.5 fold suppression or less (mean .94). Peripheral lymphocyte counts were similar in both groups. Incubation of lymphocytes with purified viral preparations are currently being evaluated.

Current studies are also under way to examine possible serum factors responsible for this suppression, as well as the ratios of B to T lymphocytes in peripheral blood during the infection.

4) Further characterization of Norwalk Agent. Previous studies had indicated that the characteristics of the Norwalk virus most closely resembled those of a parvovirus (Dolin). The particle visualized by IEM had a buoyant density in cesium of 1.40 gm /cc consistent with that of a parvovirus (Gerin). In an attempt to further establish that this particle was indeed the infectious agent, stool filtrates were treated with genetron 113, and banded in potassium tartrate. Fractions of the band in density ranges of (a) 1.5 to 1.4lgm/cc, (b) 1.37-1.28 gm/cc (c) 1.23 - to 1.0 gm/cc were prepared and administered to volunteers after dilution to their original volume. Despite the fact that the vast majority of particle was present in fraction "b"(by IEM), disease was produced by light, middle and heavy fractions in all experiments, suggesting that incomplete separation had occurred. However, clearly more severe disease occurred with the light fraction than with the others. Furthermore, a 10^{-3} dilution of the light fraction caused more severe illness than a 10^{-1} dilution, suggesting that an inhibitor may have been diluted out. This light fraction is now under intensive investigation in organ and tissue culture.

5) Possible visualization of particle etiologically associated with Hawaii ("H") agent. By use of the same IEM techniques previously applied to the Norwalk agent, a viral particle was recently visualized in the stool of a volunteer who received Hawaii inoculum (Wyatt, Thornhill). Preliminary studies are consistent with antibody rises in sera from the original outbreak, and from subsequent volunteer studies. This finding represents the second such viral particle visualized from our seven infectious inocula (Norwalk virus, being the first). and holds promise for future characterization of the H agent.

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Significance to Biomedical Research and the Program of the Institute:

Acute infectious nonbacterial gastroenteritis is a common disease which affects all age groups of a broad segment of the population. It is estimated to be the second most common disease experience in the United States in a ten year family study. Identification and characterization of etiologic agents, as well as studies of the pathogenesis of this disease in man, are first steps toward the ultimate goals of prevention and therapy. The newly developed techniques of immune electron microscopy and fetal intestinal organ cultures, as well as volunteer experiments, should continue to provide valuable data.

Proposed Course:

Intensive studies of the agents of AINBG currently available will continue. Emphasis will be placed on biophysical purification and the development of rapid, sensitive serologic assays. Efforts to propagate the agents in vitro in organ and tissue cultures will continue. The host response in man will be further investigated with particular emphasis on the role of local and systemic antibodies, interferon production, and cell mediated immunity. The possible role of these viruses in gastrointestinal diseases such as tropical sprue, regional enteritis and ulcerative colitis will be investigated.

Honors and Awards: NONE

Publications:

1. Buscho, R. F., Wyatt, R. G., Dolin, R., Blacklow, N. R., DuPont, H. and Chanock, R. M.: Recurrent outbreaks of acute infectious nonbacterial gastroenteritis. Am. J. Epidemio. 98: 192-198, 1973.

2. Wyatt, R. G., Kalica, A. R., and Dolin, R.: Ultrastructural study of viral replication in human fetal intestinal organ culture. Archiv. Virusforschung. 40: 341-349, 1973.

3. Agus, S. G., Dolin, R., Wyatt, R. G., Tousimis, A. J. and Northrup, R. S.: Acute infectious nonbacterial gastroenteritis: intestinal histopathology. Histologic and enzymatic alterations during illness produced by the Norwalk agent in man. Ann. Int. Med. 79: 18-25, 1973.

4. Agus, S. G., Falchuk, M., Sessoms, C., Wyatt, R. G., and Dolin, R.: Increased jejunal IgA synthesis in vitro during acute infectious nonbacterial gastroenteritis. Am. J. Digest. Dis. 19: 127-131, 1974.

5. Wyatt, R. G., Dolin, R., Blacklow, N. R., DuPont, H. L., Buscho, R. F., Thornhill, T. S., Kapikian, A. Z., and Chanock, R. M.: Comparison of three agents of acute infectious nonbacterial gastroenteritis by cross-challenge in volunteers. J. Infect. Dis. In press.

Serial No. NIAID-54(c)
1. LCI
2. Clinical Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Regulation of the Immune Response in Man and Experimental Animals

Previous Serial Number: SAME

Principal Investigator: Dr. Anthony S. Fauci

Other Investigators: Dr. James E. Balow
Dr. Douglas L. Hurley
Dr. Sheldon M. Wolff
Dr. David C. Dale
Ms. Kathy O'Meara

Cooperating Units: NONE

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	4-5/12			4-5/12
Professional	2-11/12			2-11/12
Other	1-6/12			1-6/12

Project Description:

Objectives:

1) To delineate the precise mechanisms whereby immunosuppressive agents such as cytotoxic drugs and corticosteroids affect the immune response in man and in experimental animals, specifically looking at in vivo and in vitro function of the monocyte and subpopulations of lymphocytes.

2) To separate and prepare purified subpopulations of lymphocytes in man and animals in order to more accurately define their functional capacity and to study their role in such disease states as hypersensitivity vasculitis, granuloma formation, tumor and graft rejection, and the graft-versus-host reaction. In particular, to characterize the sensitivity of these subpopulations to various immunosuppressive agents.

3) To study the mechanisms whereby altered states of immunity as described above affect host mechanisms against infections. Specifically, to develop an animal model of disseminated fungemia and bacteremia in a steroid-resistent species such as the guinea pig which is similar to man in its relative resistence to the lymphopenic effects of corticosteroids, and to use this model to characterize the cell populations which are specifically suppressed in states of altered immunity associated with disseminated infections.

Methods Employed:

1) A significant proportion of the methodology is centered around the *in vitro* culturing of lymphoid cells to determine their functional capabilities. The effects of *in vivo* chemotherapeutic agents as well as the effects of disease-related altered states of immunity can thus be monitored and explored.

2) Administering corticosteroids to normal volunteers and patients requiring this drug and measuring the specific effects on monocytes and lymphocyte subpopulations.

3) Labeling a subject's lymphoid cells with ^{51}chromium and reinfusing the cells back into their circulation to determine the effects of disease states and of chemotherapeutic agents on circulation patterns and viability of these cells.

4) Identifying subpopulations of lymphoid cells by their surface markers and determining the change in relative proportions of such cells in disease states and during chemotherapy. Identifying specific lymphocyte subpopulations as the cell type involved in lymphocyte-mediated disease in man and in experimentally-induced disorders in animals.

5) Determining the immunocompetence of bone marrow lymphocytes and the redistribution of immunocompetent cells which is associated with corticosteroid administration in man and guinea pigs.

6) Producing altered states of immunity in experimental animals by x-irradiation, surgical removal of lymphoid organs, or administration of immunosuppressive agents in order to determine precisely the requirement of certain factors in the immune response necessary for host defense and immunological surveillance.

Major Findings and Significance to Biomedical Research and the Program of the Institute:

1) A group of patients with Wegener's granulomatosis (a disease characterized by granulomatous vasculitis involving the respiratory tract and kidneys) are being studied. Untreated, the disease is rapidly fatal. We are currently following the largest single group of such patients in the world. The disease serves as an excellent model of a hypersensitivity state. In addition to effecting long term remissions and even cures in this formerly fatal disease by the use of cytotoxic agents (cyclophosphamide), we were able to clearly demonstrate that on our therapeutic protocol there was suppression of particular aspects of the immune response (immunoglobulin levels, newly induced delayed hypersensitivity) with sparing of other aspects (established delayed hypersensitivity, in vitro blastogenic response to mitogens, and relative proportions of subpopulations of lymphocytes). We were able to define for the first time in human studies that cyclophosphamide is more specifically directed against B rather than T lymphocytes. This degree of immunosuppression was correlated with clinical remission of disease and was not associated with an increased incidence of infection.

2) Corticosteroids were adminstered to normal volunteers in order to determine the precise effects upon the immune response. These agents were found: a) to cause a profound but transient decrease in lymphocyte and monocyte counts, b) to cause a change in the relative proportion of lymphocyte subpopulations in the circulation, and c) to cause a differential and selective depression of certain lymphoid cell functions as measured by in vitro stimulation. This is the first time that such effects have been clearly demonstrated in humans.

In addition, by reinfusing ^{51}Cr-labelled autologous lymphocytes into patients and normal volunteers, we were able to demonstrate that the specific lymphocyte subpopulation affected by corticosteroid administration is the long-lived recirculating lymphocyte. We found that lymphocytopenia occurs following corticosteroid administration not as a result of destruction of cells, but by a redistribution of lymphocytes out of the circulation into other body compartments.

3) In studies involving a large group of patients on alternate-day corticosteroid therapy, we demonstrated the specific immunosuppressive effects of this regimen at the cellular level and characterized the dichotomy between suppression of inflammatory disease activity and preservation of normal host defense.

4) In guinea pigs, we were able to characterize the specific subpopulations affected and the mechanisms of the effect of corticosteroid-induced lymphocytopenia. This is particularly relevant in light of the growing body of information linking different lymphocyte subpopulations with specific diseases.

5) We were able to establish a guinea pig model of disseminated candidiasis and subsequently we demonstrated the relative efficacy of alternate-day corticosteroid therapy versus daily corticosteroid therapy in the preservation of host defense against infection.

Honors and Awards: NONE

Publications:

1. Fauci, A. S. and Wolff, S. M.: Wegener's granulomatosis: Studies in eighteen patients and a review of the literature. Medicine, 52: 535-561, 1973.

2. Fauci, A. S. and Dale, D. C.: The effect of in vivo hydrocortisone on subpopulations of human lymphocytes. J. Clin. Invest. 53: 240-246, 1974.

3. Fauci, A. S. and Wolff, S. M.: Wegener's granulomatosis. In Schreiner, G. E. (ed.): Perspectives in Nephrology. New York, J. Wiley & Sons. In press.

4. Wolff, S. M., Fauci, A. S., Horn, R. G. and Dale, D. C.: Wegener's granulomatosis. Ann. Intern. Med. In press.

5. Fauci, A. S., Dale, D. C. and Wolff, S. M.: Cyclophosphamide and lymphocyte subpopulations in Wegener's granulomatosis. Arthritis Rheum. In press.

6. Horn, R. G., Fauci, A. S., Rosenthal, A. S. and Wolff, S. M.: Renal biopsy pathology in Wegener's granulomatosis. Am. J. Pathol. 74: 423-434, 1974.

7. Fauci, A. S.: Corticosteroids and circulating lymphocytes. Transplantation Proc. In press.

8. Fauci, A. S.: Pulmonary vasculitis. Kirkpatrick, C. H. and Reynolds, H. Y. (eds.) In Immunologic and Infectious Reactions in the Lung. New York, Marcel Dekker, Inc. In press.

Serial No. NIAID-55(c)
1. LCI
2. Clinical Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Granulocyte Physiology

Previous Serial Number: SAME

Principal Investigator: Dr. David C. Dale

Other Investigators: Dr. Michael J. Chusid
Dr. Charles A. Dinarello
Dr. Anthony S. Fauci
Dr. James E. Pennington
Dr. Herbert Y. Reynolds
Dr. Sheldon M. Wolff
Dr. John Wewerka
Mr. Stanley B. Ward
Mr. Edward W. Harvey
Ms. Rhoda T. Hubert

Cooperating Units: Dr. John Adamson, University of Washington, Department of Medicine, Seattle, WA
Dr. David W. Alling, OSD, NIAID, NIH (no project no.)
Dr. Jeffrey S. Borer, NHLI, NIH (no project no.)
Dr. Ronald J. Elin, CPD, CC (no project no.)
Dr. Robert Graw, NCI, NIH (project no. 6820)
Dr. Mitsuhiro Omine, NCI, NIH (no project no.)
Dr. Seymour Perry, NCI, NIH (no project no.)
Dr. Howard Hoffman, NICHHD, NIH (no project no.)
Dr. Emanuel Bessa, University of Pennsylvania, Philadelphia, PA
Dr. Frank Gardner, University of Pennsylvania, Philadelphia, PA

LVD
LM
LBV
LMI
LID

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	4-3/12		10/12	5-1/12
Professional	2-3/12		10/12	3-1/12
Other	2			2

Project Description:

Patients and animal studies are conducted to investigate the regulation of granulocytopoiesis and the role of granulocytes in inflammation and defense against infection.

Objectives:

Human Studies:

1. To study the factors regulating the blood granulocyte count of normal individuals and patients with infectious, inflammatory, immunologic, and hematologic diseases.

2. To determine the pathophysiologic mechanisms of chronic and cyclic neutropenia.

3. To investigate the mechanisms of the hypereosinophilic syndrome. To study the role of eosinophils in causing the peculiar tissue injury seen in this disorder.

4. To assess the effects of immunosuppressive agents on granulocyte physiology. To better define the factors of increased host susceptibility to infection caused by these agents and better understand the basic mechanisms of immunosuppression.

5. To clinically evaluate patients with prolonged unexplained fever to delineate new inflammatory diseases and host defense abnormalities.

Animal Studies:

1. To define the mechanisms of cyclic hematopoiesis in grey collie dogs. To explore in these animals methods for altering cyclic neutropenia which may be applicable to the similar human disorder.

2. To study the treatment of experimental pneumonia in leukopenic dogs. To evaluate the relationship of the blood granulocyte count to susceptibility to infection and the use of granulocyte transfusion therapy.

Methods Employed:

Granulocyte counts are measured for long periods to diagnose and investigate cyclic neutropenia and other disorders of neutrophil production.

To blood granulocyte count is measured after administration of etiocholanolone, endotoxin, cortisol and prednisone to evaluate the bone marrow granulocyte reserve.

The blood granulocyte pool sizes, distribution and turnover rates are measured by radioisotopic labeling studies employing $DF^{32}P$ and ^{51}Cr.

In vivo studies of leukocyte chemotaxis are measured by the skin window technique.

Major Findings:

1) Cyclic hematopoiesis in man and in dogs is accompanied by cyclic fluctuation of erythropoetin and colony stimulating factor. Bacteriocidal function of neutrophils in canine cyclic neutropenia is defective. Cyclic neutropenia in dogs can be cured by bone marrow transplantation.

2) Granulocyte transfusion therapy is effective treatment of experimental Pseudomonas penumonia in leukopenic dogs. Transfusions prolong survival, improve clearance of bacteria from the infected lung and prevent endotoxemia as measured by the limulus test. Granulocytes procured by the cell centrifuge or filter adherence are both effective.

3) Alternate-day prednisone therapy is accompanied by no apparent predisposition to infections. With this therapy studies of granulocyte kinetics indicate that the day off prednisone neutrophil kinetics are normal. The day on prednisone, neutrophil half-life is prolonged proportional to the prednisone dose. The cutaneous inflammatory response is reduced by daily therapy but not be alternate day therapy.

4) Etiocheolanolone has therapeutically beneficial effects for treatment of the refractory anemia of myelofibrosis. Encouraging studies of its use in other forms of hypoplastic anemia are now in progress.

5) Prednisone and cortisone have been compared with etiocholanolone and endotoxin for experimental production of leukocytosis in man. Cortisone appears to be a useful agent for study of the marrow reserve response.

6) Eosinophil kinetic studies in patients with idiopathic eosinophilia indicate that eosinophils are a recirculating population of cells with distinctly different blood kinetic from neutrophils. Chronic eosinophilia, in the absence of overt signs of heart disease, is accompanied by echocardiographic changes indicating venticular wall thickening. Except for adrenocorticosteroids which benefit some cases of hypereosinophilia, there is no known therapy for this condition. In the past year, one patient with severe hypereosinophilia has been dramatically benefited by treatment with hydroxylurea.

7) Following injection of etiocholanolone, a leukocytosis-inducing factor can be measured in the plasma of normal subjects.

Significance to Biomedical Research and the Program of the Institute:

These studies directly relate to understanding the role of granulocytes in resistance to infection.

Proposed Course:

The studies will be largely curtailed since the Principal Investigator is leaving NIH in August, 1974.

Honors and Awards: NONE

Publications:

1. Geelhoed, G. W., Kane, M. A., Dale, D. C. and Wells, S. A.: Colon ulceration and perforation in cyclic neutropenia. J. Pediatr. Surg. 8: 379-382, 1973.

2. Dale, D. C., Fauci, A. S. and Wolff, S. M.: The effect of cyclophosphamide on leukocyte kinetics and susceptibility to infection in patients with Wegener's granulomatosis. Arthritis Rheum. 16: 657-664, 1973.

3. Guerry, D. IV, Dale, D. C., Omine, M., Perry, S. and Wolff, S. M.: Periodic hematopoiesis in human cyclic neutropenia. J. Clin. Invest. 52: 3220-3230, 1973.

4. Dale, D. C. and Graw, R. G.: Transplantation of allogeneic bone marrow in canine cyclic neutropenia. Science 183: 83-84, 1974.

5. Hoffman, H. J., Guerry, D. IV, and Dale, D. C.: Analysis of cyclic neutropenia using digital band pass filtering techniques. J. Interdisciplinary Cycle Research. In press.

6. Fauci, A. S., Dale, D. C. and Wolff, S. M.: Cyclophosphamide and lymphocyte subpopulations in Wegener's granulomatosis. Arthritis Rheum. In press.

7. Guerry, D., Adamson, J. W., Dale, D. C. and Wolff, S. M.: Human cyclic neutropenia: Urinary colony stimulating factor and erythropoietin levels. Blood. In press.

8. Dale, D. C., Reynolds, H. Y., Pennington, J. E., Elin, R. J., Pitts, T. W. and Graw, R. G.: Granulocyte transfusion therapy of experimental pseudomonas pneumonia. In Proceedings of the Conference on Pseudomonas Infections. Washington, D. C. Walter Reed Army Institute of Research. In press.

LVD
LM
LBV
LMI
LID

Serial No. NIAID-56(c)
1. LCI
2. Allergic Diseases Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Biochemical Pathways of Mediator Release and Mechanism of Tissue Injury in Allergic Diseases

Previous Serial Number: SAME

Principal Investigator: Dr. Allen P. Kaplan

Other Investigators: Dr. John I. Gallin
Dr. Louis W. Heck
Dr. Richard A. Clark
Dr. Lewis D. Yecies
Mr. Henry L. Meier
Mrs. C. Linda Gray
Dr. Charles H. Kirkpatrick
Dr. Eric A. Ottesen
Dr. Franklin A. Neva

Cooperating Units: Dr. Guillermo Pacheco, LPD, NIAID, NIH (Project No. 121J)
Dr. Fletcher Taylor, Head Allergic Diseases Section, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	5-1/12	-	-	5-1/12
Professional:	3-1/12	-	-	3-1/12
Other:	2	-	-	2

Project Description:

Objectives:

1. Determination of the interrelationship of the coagulation, fibrinolytic, kinin-generating, and complement pathways in human plasma. The relevant proteins will be purified, their mechanism of activation determined, and their role in the various enzymatic pathways described. Vasoactive peptides or chemotactic factors released as a consequence of activation of the coagulation and fibrinolytic pathways will be defined. The role of plasma inhibitors to control each of these pathways will be determined.

2. Development of radioimmunoassays or radial immunodiffusion assays for assessment of the bradykinin forming pathway in human plasma focusing attention upon the early steps of the intrinsic coagulation and fibrinolytic pathways with which it is intimately associated.

3. Determination of the mechanism of interaction of the chemotactic enzymes kallikrein and plasminogen activator with the surface of the neutrophil and monocyte.

4. To identify the chemotactic factors which are generated by activation of the Hageman factor dependent pathways in human plasma or serum.

5. To improve the methodology for the separation of prekallikrein from plasminogen proactivator, two very closely associated substrates of activated Hageman factor and compare the mechanism by which human plasma plasminogen activator converts plasminogen to plasmin with known plasminogen activators such as urokinase and streptokinase.

6. Investigation of the pathogenesis of cold urticaria, cholinergic urticaria, and chronic ideopathic urticaria and angioedema, specifically focusing upon identification of the vasoactive principle which causes the defect, identifying any immunologic mechanism which may play a role, and defining the biochemical pathway which is activated in each disorder. Particular attention is given to any role played by IgE antibody, the human basophil and mast cell, and the possibility of a platelet activating factor liberated by the basophil causing secondary degranulation of platelets with its attendant seratonin release.

7. Investigation of the role of thymic-dependent lymphocytes in initiating IgE synthesis and subsequently to cause IgE synthesis to cease (thymic suppressor cells) (in collaboration with Drs. Kirkpatrick and Ottesen). An abnormality in the latter function may be responsible for the heightened IgE response characteristic of "atopic" individuals who have hay fever or asthma. Any soluble mediator released from the T cell which may play a role in IgE suppression will be isolated and characterized.

Methods Employed:

Purification of plasma proteins is performed utilizing affinity chromatography, ion exchange chromatography on Quarternary aminoethyl (QAE) Sephadex and Sulphopropyl (SP) Sephadex, gel filtration, and preparative elution from gels after isoelectric focusing. These methods have been applied to the isolation of unactivated Hageman factor, Hageman factor prealbumin fragments, pre-PTA (coagulation factor XI), prekallikrein, plasminogen proactivator, plasminogen and the plasma inhibitors α_1 antitrypsin, α_2 macroglobulin, the inhibitor of the activated first component of complement ($C\bar{1}$ INH), inter α trypsin inhibitor, α_1 chymotrypsin inhibitor, and antithrombin III. Purity is evaluated by alkaline disc gel electrophoresis, SDS gel electrophoresis, and isoelectric focusing.

The chemotactic activity generated by kaolin activation of human serum or plasma is assessed utilizing the radioassay described by Gallin et al. (J. Immunol. 110: 233, 1973). Mononuclear chemotaxis was performed by the Standard Boyden Chamber assay utilizing partially purified mononuclear cells by Hypaque-Ficoll gradient. Plasma histamine and seratonin levels are determined

by the radioenzyme method. Histamine release from leucocytes is determined by either fluorometric assay or radioenzyme assay. Plasma bradykinin and IgE levels are determined by radioimmunoassay.

Major Findings:

1. The Hageman factor substrates pre-PTA have been isolated free of any contaminating proteins by sequential chromatography on QAE Sephadex, SP Sephadex, Sephadex G150 and passage over an anti-IgG immunoabsorbant. It was found to possess a molecular weight of 175,000 by Sephadex gel filtration, 170,000 by SDS gel electrophoresis, and an isoelectric point of 9.1. It could be activated directly by activated Hageman factor or Hageman factor fragments. The major plasma inhibitor of activated PTA was found to be α_1 antitrypsin; the $C\bar{1}$ INH and antithrombin III, both previously described PTA inhibitors, were also positive but possessed less activity.

2. The chemotactic enzymes kallikrein and plasminogen activator were also found to be chemotactic for human mononuclear cells. The chemotactic activity was inhibited if the active site of the enzymes was destroyed with DFP. However, DFP treatment of the respective proenzymes did not prevent their subsequent activation by Hageman factor to chemotactic enzymes. The natural inhibitors of each enzyme were also found to inhibit their chemotactic activity, hence, α_2 macroglobulin inhibited the chemotactic activity of both kallikrein and plasminogen activator. However, $C\bar{1}$ INH inhibited only the chemotactic activity of kallikrein.

3. Further studies of Fletcher factor deficiency have indicated that the defect is due to prekallikrein deficiency and that the active enzyme kallikrein affects coagulation and fibrinolysis by activating Hageman factor by a feed-back mechanism. However, we have shown that activated Hageman factor bypasses this feedback and corrects the deficiency. However kallikrein, when added to prekallikrein deficient plasma in the absence of kaolin (which slowly activates Hageman factor), does not correct the deficiency by activation of Hageman factor in the fluid phase although utilizing purified components, this appears to be a rapid reaction. Thus the initial step of intrinsic coagulation appears to require binding of Hageman factor to an appropriate surface, activation of the Hageman factor, activation of some fluid phase prekallikrein to kallikrein, and then further recruitment of Hageman factor by kallikrein acting upon the bound Hageman factor prior to any further recruitment of Hageman factor by its enzymatic activation in the fluid phase.

4. Administration of endotoxin to normal volunteers has shown to generate measurable quantities of kallikrein and plasminogen activator as assessed by their kinin-generating and fibrinolytic functions respectively as well as their chemotactic functions. A low molecular weight chemotactic factor has also been shown to be generated by this in vivo challenge which does not correspond to the known complement dependent chemotactic factors C5a or C3a which are readily demonstrable after in vitro activation of serum. This new factor is dependent upon Hageman factor for its generation and appears to be a chemotactic peptide generated via the coagulation pathway. Its identification and

mechanism of generation are being studied.

5. Study of the pathogenesis of cold urticaria has demonstrated that most patients have an IgE mediated mechanism such that the defect can be passively transferred to normal volunteers and successfully challenged with an ice cube 48 hours later. Passage of sera over an anti-IgE immunoabsorbant removes this activity. Elation of the IgE from those columns to study the mechanism of its interaction with the basophil and/or mast cell are in progress. By radioenzyme assay histamine has been shown to be a major mediator of the swelling in those patients as evaluated by submersion of their hand in ice water for five minutes and determining serial histamine levels in the draining brachial vein over a 20 minute period. Thus far determination of seratonin and bradykinin have been negative.

6. Patients with tropical eosinophilia, a parasitic disease apparently caused by a filarial organism present with patchy pulmonary infiltrates, asthma, and eosinophila. These patients have been shown to possess significantly elevated IgE levels when compared to patients with filariasis who have no pulmonary symptoms. Hemagglutinating antibody is similarly elevated. It is possible that those patients with tropical eosinophilia have a heightened immune response to this organism, clear it from the circulation, and deposit them in the lung where an inflammatory reaction is triggered. Studies are now being continued to determine whether this IgE antibody is specifically directed toward filarial antigens. Various filarial antigens are being prepared; a positive reaction might be useful in determining the specific etiologic agent (or agents) in this disorder.

Significance to Biomedical Research and the Program of the Institute:

The interrelated enzymatic pathways consisting of coagulation, fibrinolysis, and kinin-generation have not been extensively studied in human disease. The role played by these enzymes and their vasoactive and chemotactic by-products are being evaluated in various types of chronic urticaria and angioedema, cold urticaria, cholinergic urticaria, and urticarial erythema multiforme as one aspect of a larger program being developed in the LCI devoted to the investigation of the etiology, pathogenesis, and therapy of allergic diseases. Hopefully identification of the various mediators and enzymatic pathways activated in these disorders will lead to an understanding of their pathophysiology and hopefully to new modes of therapy.

Proposed Course:

To continue the studies outlined above and develop the ability to evaluate all of the mediator systems in patients with allergic diseases. The mechanism of activation of Hageman factor and its substrates, the inhibition of their activities by natural inhibitors, and the generation of chemotactic factors will be pursued. Assays will be developed to measure each factor functionally and as a protein in human plasma. The role of IgE, the basophil, and the tissue mast cell in human allergic diseases will be evaluated with the emphasis upon various types of urticarias and angioedemas.

Honors and Awards: NONE

Publications:

1. Weiss, A. S., Gallin, J. I., and Kaplan, A. P.: Fletcher factor deficiency: A diminished rate of Hageman factor activation caused by absence of prekallikrein with abnormalities of coagulation, fibrinolysis, chemotactic activity, and kinin generation. J. Clin. Invest. 53: 622-633, 1974.

2. Kaplan, A. P.: The Hageman factor dependent pathways of human plasma. Microvasc. Res. In press.

Serial No. NIAID- 57 (c)
1. Laboratory of Clinical Investigation
2. Clinical Mycology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Basic studies on pathogenic fungi

Previous Serial Number: 77-1F and 77-1-G

Principal Investigator: Dr. K.J. Kwon-Chung

Cooperating Units: Dr. S.J. Kim, Laboratory of Microbiology, NIAID
(No Project number)
Dr. G. W. A. Milne, Laboratory of Chemistry, NHLI
(No Project No.)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	3			3
Professional	1			1
Other	2			2

Project Description:

Objectives:

Objectives of these studies cover both basic and applied aspects of various pathogenic or nonpathogenic fungi, including their morphology, taxonomic position, genetics and relationship between their sterol content and resistance to antifungal antibiotics.

Methods employed:

Morphological studies were carried out by culturing the fungi on various mycological media at different temperatures. Observations were made with a light microscope with or without Normarsky interference condenser. For genetic studies of Histoplasma capsulatum (Emmonsiella

capsulata), two tester strains (-,+) were crossed on alphacel yeast extract agar and incubated for 3-4 weeks and the F_1 progeny were obtained by single ascospore isolation. All F_1 strains were cultured on blood glucose cysteine agar and Pines medium at 37°C to convert them into their unicellular forms. The F_1 strains were also cultured on soil extract agar for observations of their colonial characteristics. The serotypes of parent strains as well as F_1 strains were determined by reciprocal cross-staining and absorption using fluorescein-labeled reagents.

Eighteen clinical isolates of Histoplasma duboisii were obtained from various countries of Africa. They were crossed in all possible combinations and also crossed with two strains of H. capsulatum to determine their genetic relationships.

Mutants resistant to various polyene antibotics were obtained using both mating types of Aspergillus fennelliae and one strain of Cryptococcus neoformans. The mutation was induced either by ultraviolet irradiation or by culturing the organism on drug containing media for many generations. Sterols from wild types as well as mutants were extracted and purified by conventional chemical methods. Cross resistance of mutants to a variety of polyene drugs was tested. Identification of new sterols from mutants was carried out using gas liquid chromatography, UV spectrophotometry, nuclear magnetic resonance, infrared spectrometry and mass spectrometry. Pathogenicity of mutants and the wild type of C. neoforms was tested by inoculating various number of cells into mice and observing mortality rates.

Major Findings:

A. Genetics of Histoplasma capsulatum

1. Conversion of this fungus from mycelial to yeast on blood-glucose-cysteine agar medium was extremely poor compared to that on Pine's agar. Tests on 101 F_1 strains obtained by crossing the strain T-2-1 (easily converting) and T-4-2 (hard to convert) showed that mating type had no relationship with in vitro covertability. This was contrary to expectation.

2. Serotype of H. capsulatum was found to be genetically determined. F_1 progeny obtained by crossing the 1, 2 type and the 1,2,3 type were either 1,2 or 1,2,3 type. All 68 progeny obtained from a pair of 1,2,3 types were 1,2,3 type.

3. Crosses of 18 clinical isolates of H. duboisii with tester strains of H. capsulatum indicated that the two fungi belong to one species and that "—" type is 5 times more frequently isolated than the "+" type from African histoplasmosis cases.

B. Polyene resistance and sterol content of Aspergillus and Cryptococcus neoformans.

Aspergillus fennelliae:

1) After 32 successive transfers of the wild type strain of Aspergillus

fennelliae AF5 (A mating type) on a series of amphotericin B media and 18 serial transfers of AF4 (a mating type) on nystatin media resulted in drug resistant mutants AF5-AB1 and AF4-NS1 respectively. Approximately 100 fold increase of resistance to amphotericin B was observed in AF5-AB1 and 4 fold increase of resistance to nystatin was observed in AF4-NS1.

2) Sterol analyses of wild types and the mutants showed that ergosterol was the major sterol of the wild types while two new compounds were the major sterols for the mutants. The strain AF5-AB1 lacked ergosterol but AF4-NS1 retained a minor fraction of ergosterol. One of the new sterols was identified as $\Delta 7,22$ ergostadiene-3β-ol which has not been reported in the filamentous fungi.

3) Growth studies revealed that the mutant lacking ergosterol, AF5-AB1 manifested reduced growth rate and poor sporulation. The mutant AF4-NS1 which retained a fraction of ergosterol showed almost the same rate of growth and sporulation as the wild type.

4) The F_1 progeny obtained by crossing mutants and wild types showed that: (a) heavy conidial and poor conidial types in this species are controlled by a single locus consisting of two alleles which segregate independently of the locus for mating type, (b) the amphotericin B resistant mutant and the nystatin resistant mutant are due to single mutations, (c) the sensitivity to the drug is directly related to the level of ergosterol and (d) synthesis of ergosterol is also directly related to sexual and asexual reproduction.

5) Mutants resistant to amphotericin B showed an increased minimal inhibitory concentration (MIC) for nystatin, pimaricin and filipin while the mutants resistant to nystatin conferred increased MIC for pimaricin and filipin but not for amphotericin B.

Cryptococcus neoformans

1) All of the nine drug resistant strains of C. neoformans, obtained by irradiating (U.V.) two wild type strains, showed altered sterol content. Thus far identified sterols are $\Delta 7,22$, ergostadiene - 3β-ol and $\Delta 7$ ergostenol-3β-ol from 4 mutants resistant to amphotericin B and nystatin, and $\Delta 8$, ergostenol from 2 pimaricin resistant mutants. The two sterols $\Delta 7$, ergostenol-3β-ol and $\Delta 8$, ergostenol have not been reported in fungi.

2) Preliminary study showed that all resistant mutants are considerably less virulent than the wild types.

C. Discovery of a new species of Aspergillus pathogenic to man.

Two strains of Aspergillus, one isolated from a patient (biopsy material) suffering from sphenoid sinusitis and the other isolated from bronchial washing of another patient, showed several distinct features not found in any other known species of Aspergillus. Over all morphological characteristics of these strains belong to those of Aspergillus fumigatus series. However, in addition to the formation of restricted colonies on standard media, persistantly septated phialids were produced in these strains. Phialids were twice longer than the strains of A. fumigatus and conidiophores were also frequently septated. The new species produced smooth, pyriform conida compared to the echinulated spherical spores of A. fumigatus. The

optimum temperature for growth was 37°C as in strains of A. fumigatus. Colonies were cream to rose colored, in contrast to green colonies of A. fumigatus grown the same temperature. Unlike A. fumigatus, the new species failed to grow on agar media containing more than 20% sucrose or dextrose. Experimental infection in mice showed that the new species was equally or more pathogenic than strains of A. fumigatus.

Significance to Biomedical Research and the Program of the Institute:

Our major effort has been directed toward the investigation of the relationship between polyene drug resistance and the sterol content in a filamentous fungus, Aspergillus fennelliae, and in a pathogenic yeast, Cryptococcus neoformans. Genetic bases of sterol synthesis and the drug resistance was analyzed using the Aspergillus species. This information will elucidate the mechanism of drug resistance as well as the biosynthesic pathway of sterols in fungi.

We have also directed our effort toward investigations of the genetic relationship between mating types, dimorphism, and serotypes of Histoplasma capsulatum in an effort to assess their basic biological characteristics. This information will be helpful in understanding the pathogenicity of the species.

Discovery of a new species of Aspergillus pathogenic to man broadens the scope of our knowledge on the etiology of aspergillosis.

Proposed Course:

Further studies are being directed to investigate the relationship between mating type and dimorphism of H. capsulatum in vivo. Methods are being tested that will enhance both germination of ascospores and colony formation of yeast cells of the same organism. The two main obstacles to extensive genetic studies in H. capsulatum have been low germination rates of ascospores, and low rate of colony formation by yeast cells.

The effect of environmental differences on mutations leading to sterility in H. capsulatum will be investigated using natural or artificial culture media at various temperatures.

Continued efforts will be directed towards the identification of several unknown sterols isolated from the polyene resistant mutants of Aspergillus fennelliae and Cryptococcus neoformans. The relationship between reverse mutation and sterol content will also be investigated.

Critical comparison will be made between isolates of Sporothrix schenckii and Ceratocystis stenoceras to clarify the existing confusion on the sexual state of S. schenckii.

Honors and Awards:

Member, Nomination Committee for Medical Mycological Society of America.

Chaired a Medical Mycology Section at Annual Meeting of American Society for Microbiology, 1974, in Chicago.

Publications:

1. Young, N. A., Kwon-Chung, K. J., and Freeman, J.: Subcutaneous abscess caused by Phoma sp. resembling Pyrenochaeta romeroi: Unique fungal infection occurring in immunosuppressed recipient of renal allography. Am. J. Clin. Pathol. 59: 810-916, 1973.

2. Wong, V. G., Kwon-Chung, K. J., Green, W. R., Anderson, R. R., Collins, E. M., and Hill, W. B.: Focal choroidopathy in experimental ocular histoplasmosis. Trans. American Academy of Ophthalmology and Otolaryngology. 77: 769-777, 1973.

3. Kwon-Chung, K.J., Weeks, R. J., and Larsh, H.W.: Studies on Emmonsiella capsulata (Histoplasma capsulatum). II. Distribution of the two mating types in 13 endemic states of the United States. Amer. J. Epidemiol. 99: 44-49, 1974.

4. Bujak, J. S., Kwon-Chung, K. J., and Chusid, M. J.: Osteomyelitis and pneumonia in a boy with chronic granulomatous disease of childhood caused by a mutant strain of Aspergillus nidulans. J. Clin. Pathol.61: 361-367, 1973.

5. Kwon-Chung, K. J.: Genetics of fungi pathogenic for man. Critical Rev. Microbiol. 3: 115-133, 1974.

6. Kwon-Chung, K. J. and Kim, S. J.: A second heterothallic Aspergillus. Mycologia. In press.

Kim, S. J. and Kwon-Chung, K. J.: Polyene resistance in the sterol mutants of Aspergillus fennelliae. Antimicrob. Agents Chemother. In press.

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Serial No. NIAID-58(c)
1. LCI
2. Medical Virology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Pathogenesis and Chemotherapy of Herpes Virus Infections in Man

Previous Serial Number: NONE

Principal Investigator: Dr. Raphael Dolin

Other Investigators: Dr. Millicent Coker
Mr. John Owens
Mr. Holly A. Smith

Cooperating Units: Dr. Arthur S. Levine, POB, MD, DCT, NCI (No project no.)
Dr. Vincent T. DeVita, Jr., MD, MB, NCI (No project no.)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	1- 6/12			1- 6/12
Professional	10/12			10/12
Other	8/12			8/12

Project Description:

Objectives:

1) To determine the pathogenesis, host defense mechanisms, and natural history of herpes virus infections in man, in both host-defense compromised and intact hosts.

2) To evaluate the efficacy of anti-viral agents in the treatment of herpes virus infections in man. These viruses include herpes simplex, herpes zoster, cytomegalovirus and EB virus.

Methods Employed:

1) Herpes viruses are isolated from patients referred to the medical virology section from the Clinical Center, Bethesda Naval Medical Center, and local practitioners. The viruses are isolated and characterized in human diploid fibroblasts, and in human fetal tracheal and intestinal organ cultures.

2) The effect of individual and combined anti-viral drugs such as ARA-A, ARA-C, and IUdR on clinical isolates are studied in tissue and organ culture assays.

3) Host defense responses to herpetic viral infections are examined in the above patients. These include a) levels of circulating and locally produced neutralizing and complement fixing antibodies; b) cell mediated immune responses, i.e., skin test sensitivity, mitogen stimulations of peripheral lymphocyte cultures, and circulating B to T cell proportions; c) systemic and local (e.g. vesicle) interferon production.

4) The clinical efficacy of ARA-A in the treatment of herpes zoster and herpes simplex infections in the immunosuppressed is investigated in a randomized, controlled, double blind study, in collaboration with an NIAID supported nation-wide trial of which the principal investigator is the project officer. The efficacy of ARA-A and of IUdR in herpex simplex encephalitis and in neo-natal herpes infections is studied in a similar trial.

Major Findings:

1) Establishment of anti-viral assay. A rapid, reliable, sensitive bio-assay for anti-viral activity has been established. Anti-viral drug levels can be determined within 24 hrs of receipt of a specimen, to levels of 0.5 µg/ml of ARA-A and 0.05 µg/ml of IUdR. This assay is thus 24-48 hrs more rapid and 10 fold more sensitive than those previously described in the literature. Employing a rapidly growing and plaque forming strain of herpes simplex virus type I ("F" strain) isolated at the LCI in December '73, 80-100 plaque forming units (PFU) are inoculated into Baby Hamster Kidney tissue culture monolayers. The virus is allowed to adsorb for 2 hrs, and then the fluid containing anti-viral activity is added and washed off. The cultures are overlayed with agar, and plaques are read at 24 hrs. Previous assays of anti-viral drug activity in body fluids were confused by the presence of additional anti-viral substances such as interferon and neutralizing antibody. The use of heterologous species cultures (interferon is species specific) and non neutralizable intracellular virus circumvents these problems. This assay is currently being employed to measure anti-viral levels in body fluids and tissue compartments in patients and experimental animals; and it holds promise in providing clinically relevant information for the use of these agents.

2) Sensitivity of clinical isolates. Employing a 50% reduction in PFU as the minimum inhibitory concentration, mean MIC's against HSV I were 0.08 µg/ml of IUdR and 0.72 µg/ml of ARA-A. HSV type II appeared to be more resistant to both drugs, with a MIC of 0.35 µg/ml for IUdR and 1.1 µg/ml for ARA-A. Preliminary data suggested that ARA-A and IUdR had synergestic activity against both HSV I and II. This fact may prove clinically useful in that lower concentrations of both drugs may be used to decrease possible toxicities.

3) Evaluation of anti-viral activity in organ culture. In contrast to the de-differentiated cells of tissue culture, organ cultures theoretically provide an in vitro culture medium more representative of the in vivo conditions in man. However, no evaluation of anti-viral drugs in organ culture have been reported. Vaccinia, HSV I and II were shown to grow for the first time in tracheal organ culture, and vaccinia for the first time in intestinal organ cultures. (HSV I and II had been previously reported-Dolin). Concentrations of ARA-A up to 100 µg/ml and IUdR up to 150 µg/ml were not cytotoxic to the cultures, and did not interfere with ciliary motion on the respiratory epithelium or with smooth muscle contraction of the gut. Concentrations of 10 µg/ml of ARA-A and 50 µg/ml of IUdR inhibited HSV I and vaccinia virus growth in both intestinal and tracheal organ cultures. Inhibition of viral growth could be read directly from cytopathic effect in the organ culture, or from subsequent tissue culture assays of fluid from the organ explants. These techniques hold promise for evaluating the effect of anti-viral drugs in the pathogenesis of herpes tracheitis and esophagitis which are being reported with increasing frequency in host compromised patients. These methods can also be employed to evaluate anti-viral activity against those viruses which grow only in organ culture (e.g., the human gastroenteritis agents).

4) Animal model for herpes virus infections. Attempts to establish a systemic, pathogenic herpes simplex viral infection of guinea pigs were begun. Despite the fact that no consistent such viral infection has been previously reported in that species, the guinea pig model was selected because of the unique combination of two features among common laboratory animals: (1) skin sensitivity to antigens as an in vivo correlate to in vitro measurements of cell mediated immunity; and (2) the availability of genetically deficient animals to study the role of complement in these infections.

After adaptation of human HSV II through several passages in guinea pigs, local infection could be readily achieved but systemic infection was rare (<5%) in sero negative animals, despite administration of $10^{7.5}$ $TCID_{50}$ of virus. When systemic infection occurred, high titers of virus were found in brain, lung, liver and heart, and typical herpes inclusions were seen in histopathology. After establishing a dose response curve for lymphopenia after cytoxan administration, immunosuppressed guinea pigs are now being infected with HSV virus.

5) Clinical trials of Efficacy of ARA-A in treatment of severe herpetic infections in the immunosuppressed.

To this date 6 patients with herpes zoster have been studied in the ARA-A trial, and 1 patient with disseminated varicella has been treated with ARA-A outside of the trial. ARA-A was administered at doses of 10 mg/kg/day for five days intravenously without significant toxicity. One of the patients had mild vomiting, and one had a skin rash after 4 days of treatment necessitating cessation of drug. Five of six of the zoster patients had cutaneous anergy, and one of these went on to disseminated zoster. All six recovered from their zoster, but the patient with disseminated varicella died, 48 hours after treatment. Levels of antibody, interferon, ARA-A, as well as the effect of treatment on virus excretion and resistance are currently being evaluated.

Significance to Biomedical Research and the Program of the Institute:

Herpes viral infections are a significant and widespread cause of morbidity in normal adults and children, and a cause of significant mortality in neo-nates and the immunosuppressed. Herpes simplex encephalitis is the most common fatal encephalitis in the United States. Herpes simplex type II infections have been associated with cervical and gastrointestinal cancer. There is currently no treatment of established efficacy for these infections. Studies of the pathogenesis and host response in these infections should further define their risk to man, as well as to suggest control measures. The availability of compounds such as ARA-A which possess anti-herpes activity *in vitro* and which are relatively non-toxic in man, offer a promising modality for the treatment of these infections. Studies with this agent can serve as models for the evaluation and use of anti-viral drugs which may be subsequently developed.

Proposed Course:

Clinical trials of anti-viral agents will continue and be expanded. Host response in these patients will be further investigated. The clinical pharmacology of anti-viral drugs will be studied employing *in vivo* bioassays. The sensitivity of further isolates of HSV, herpes zoster, CMV and EBV will be determined, along with the mechanism of action of these drugs against these viruses. Efforts will continue to establish appropriate animal models to examine pathogenesis, complement activation, and immunosuppression in herpes virus infection.

Honors: NONE

Publications: NONE

Serial No. NIAID-59(c)
1. LCI
2. Medical Virology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Biology of Human Parvoviruses (Adenovirus-associated Viruses)

Previous Serial Number: NONE

Principal Investigator: Dr. Raphael Dolin

Other Investigators: NONE

Cooperating Units: Dr. M. David Hoggan, LVD, NIAID, NIH (No project no.)
Dr. Alan Rabson, LP, NCI, NIH (No project no.)
Dr. James Rose, LBV, NIAID, NIH (No project no.)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	11/12			11/12
Professional	3/12			3/12
Other	8/12			8/12

Project Description:

Objectives:

1) To study the biological and immunological properties of Adenovirus-associated viruses (AAV).

2) To investigate the relationship between AAV and its helper viruses, adenovirus and herpesvirus.

3) To study the natural history of AAV infection in man, its possible role in human disease, and particularly in adenoviral and herpesvirus infection.

Methods Employed:

The AAV are small defective viruses that are able to replicate completely only when coinfected with helper adenovirus. Patial replication (production of fluorescent stainable antigen) occurs when cultures are infected with a variety of Herpesviruses. AAV infection in man appears to be widespread since 50 to 70% of young adults possess neutralizing antibodies against AAV type 1, 2, and 3. However, infection with AAV has not been etiologically associated with human disease.

A. Assay methods - Human embryonic kidney cells (HEK), Hep-2 cells, or K-B cells are infected with AAV and helper virus. Since AAV does not produce cytopathic effects, the viruses are assayed primarily by immunologic techniques. AAV specific antigens are detected by complement fixation (CF) and immunofluorescence (FA) tests. The presence of antigen is employed as an end point in infectivity titrations.

B. Biophysical Techniques: Purified AAV, free of detectable adenovirus, is prepared by isopycnic gradient centrifugations in cesium chloride. This preparation serves as the antigen in CF tests and in the hyperimmunization of guinea pigs to produce monospecific immune sera.

C. Growth under restrictive conditions. Temperature sensitive mutants are grown under water baths of constant temperature (±.02°C) at permissive and restrictive temperatures. Plaque assays are employed to control for reversion or leakiness of the mutants.

Major Findings:

A. Preparations of mono specific sera: AAV 1, AAV 2, and AAV 3 were grown with adenovirus 7 in HEK cells. After heating at 56°C for thirty minutes, the AAV's were triply banded in cesium chloride, and the fraction with a bouyant density of 1.40 gm/cc was collected, dyalyzed against saline, and used to hyperimmunize guinea pigs. High titered sera, with minimal cross reactions in CF and FA, were prepared to each sero type.

B. Helper effect of "oncogenic" herpes viruses of primates. Epstein-Barr Virus (EBV) and Herpes Saimiri, an oncogenic virus of monkeys, provided incomplete helper functions for AAV, similar to that of other herpes viruses. The percentage of cells containing herpes-virus antigen appeared to be similar to that containing AAV antigen, when high multiplicities of infection of the latter were used. FA assays employing differentially conjugated anti-sera (Rhodamine and fluorescein) are being undertaken to attempt to demonstrate both antigens in the same cell. Growth of cells in arginine deficient media inhibits herpes virus replication and blocks the helper effects of EBV and H. Saimiri.

C. Mechanisms of Adenovirus Helper Function for AAV. Previous studies with Dr. J. Rose have demonstrated close temporal and kinetic correlation between Adenovirus DNA replication and AAV DNA replication, as well as between RNA replication in both viruses. In an attempt to separate these functions, temperature sensitive mutants of Adeno 31 were grown with AAV in HEK cells. AAV was not potentiated at the restrictive temperature when the mutant did not leak (when no infectious adenovirus was produced). Similar experiments with an adeno 5 mutant in KB cells, however, demonstrated equal concentrations of AAV ($10^{7.5}$ TCID 50/ml) at both the restrictive and permissive temperature. Since 1% Adenovirus DNA was produced at the restrictive temperature (Strauss, Rose), there appeared to be a dissociation between DNA replication in the defective and helper viruses. Further experiments employing other mutants, particularly AD_2-SV_{40} hybrids with adenoviral genome deletions are planned to clarify these findings.

D. Effect of anti-viral agents on helper-defective virus interaction. Cytosine arabinoside in concentration of 20 µg/ml inhibited Adeno 7 and HSV replication, and abolished their helper functions. Adenine-arabinoside (ARA-A) at concentrations of 10 µg/ml inhibited HSV replication and similarly abolished helper effects. ARA-A in concentrations of up to 100 µg/ml did not inhibit Adeno 2, 3, 4 or 7 replication. Its effects on AAV are currently being evaluated. Similar studies are planned for IUdR.

Significance to Biomedical Research and Program of the Institute:

Though AAV viruses commonly infect man, their significance to human disease remains unknown. Because of the pathogenic and oncogenic potential of adenoviruses and herpes viruses, their relationship to AAV may be of medical importance. These defective viruses also provide an unique opportunity to study the biology of viral interactions, providing a powerful tool to investigate both helper and defective virus functions. Furthermore, the suggestion that two recently discovered human pathogenic viruses (Hepatitis A and Norwalk viruses) most closely resemble the parvovirus group among animal viruses, suggests that an understanding of AAV biology may aid in cultivation and characterization of those hitherto undetected agents.

Proposed Course:

The potential role of AAV in human disease, acting by itself or in connection with its helper viruses, will continue to be investigated. The mechanisms of helper functions of adenoviruses and herpesviruses will also continue to be studied, with particular emphasis on the use of conditional mutants of the helper viruses. The effect of anti-viral compounds on AAV-helper virus interaction will also be examined.

Honors and Awards: NONE

Publications:

1. Dolin, R. and Rabson, A. S.: Herpesvirus Saimiri: Enhancement of Adenovirus-associated virus. J. Natl. Cancer Inst. 50: 205-207, 1973.

2. Carter, B. T., Koczot, F. J., Garrison, J., Rose, J. A., and Dolin, R.: Adenovirus-associated virus multiplication: separate helper functions provided by adenovirus. Nature 244: 71-73, 1973.

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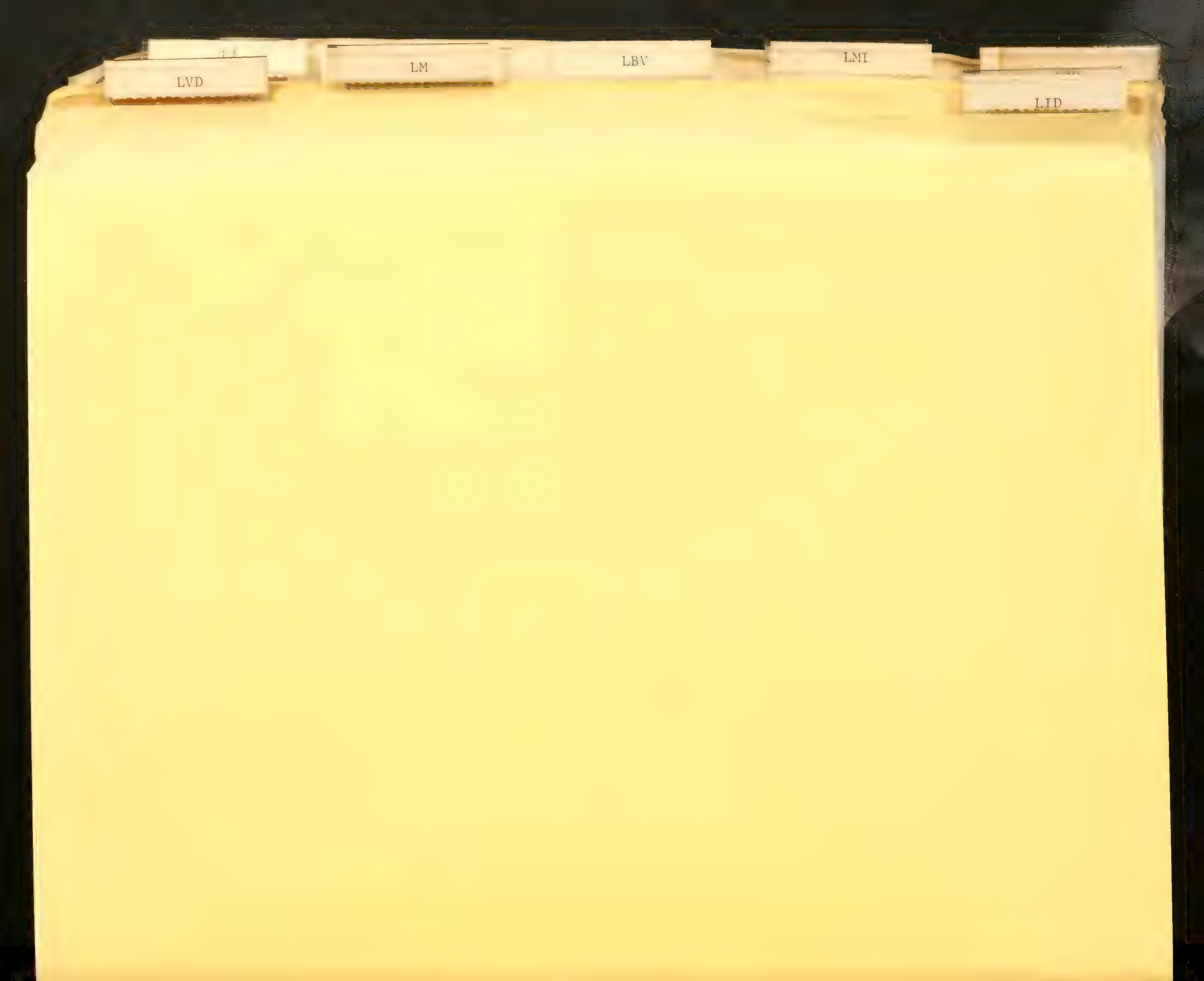
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LABORATORY OF INFECTIOUS DISEASES

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SUMMARY STATEMENT - Annual Report of the Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases
July 1, 1973 through June 30, 1974

Currently, as in the past, the research programs of LID are concerned with the natural history and prevention of three of the major uncontrolled infectious diseases of man--acute respiratory tract disease, viral hepatitis and acute gastroenteritis. Specifically, efforts are made to recover the microbial agents responsible for these diseases and to characterize the etiologic viruses or mycoplasmas in the laboratory. After techniques for the isolation of the etiologic agents and measurement of specific antibody have been developed, an attempt is made to study the natural history of infection, i.e., when and where does infection occur, what is the risk of disease during infection, and which environmental and host factors influence infection and expression of disease? Efforts are made to define the immunologic mechanisms by which the host protects himself from infection and disease. Finally, information which emerges from the foregoing studies is used to divise a strategy for prevention of illness. Special emphasis is given to the use of genetic techniques for the development of attenuated viruses and mycoplasmas which can be used for the prevention of infectious diseases of public health importance.

Acute Non-bacterial Gastroenteritis:

This program which began 4 years ago moved into a logarithmic growth phase during the past year. In previous studies a 27 nm virus particle was visualized by immune electron microscopy in the stools of individuals experimentally infected with the Norwalk agent of acute, epidemic, non-bacterial gastroenteritis. Using immune electron microscopy (IEM) we showed that persons infected under natural or experimental conditions with the Norwalk agent developed serum antibody to the 27 nm virus particle. These findings and other evidence suggested that the 27 nm virus was the etiologic agent of Norwalk disease.

To further define the 27 nm particle as the etiologic agent of the Norwalk, Ohio outbreak we examined the relationship between the appearance of the Norwalk particle in stool during the course of experimental infection and the onset of symptomatic illness. A total of 77 stools from 23 volunteers who developed gastrointestinal illness were examined by immune electron microscopy. The 27 nm virus was visualized in stool filtrates shortly after the onset of clinical illness but we failed to find it before the onset of clinical symptoms or after recovery from illness. These observations provided further support for the view that the 27 nm particle was the etiologic agent of the Norwalk epidemic of acute gastroenteritis.

The Norwalk agent was administered by stomach tube to chimpanzees in an attempt to induce experimental disease in this species. A significant serum antibody response to the 27 nm Norwalk virus-like particle was demonstrated in all animals, using immune electron microscopy. Overt clinical illness, characterized by diarrhea and/or vomiting, did not occur. Transmission of the infection was successfully accomplished by feeding additional chimpanzees a stool filtrate prepared from one of the previously challenged animals. This animal model should

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facilitate further investigations with viral agents of acute gastroenteric disease. Rhesus monkeys, baboons, and calves did not develop serum antibody responses when given the same filtrate orally.

Until recently, we had succeeded in visualizing a small entero- or parvovirus-like particle only in stools from individuals infected with the agent of Norwalk gastroenteritis although we had examined material from a number of outbreaks. Therefore, we were excited to find a 27 nm virus particle in stool filtrates from persons who became ill after receiving the Hawaii strain of acute non-bacterial gastroenteritis.

When the filtrates containing this particle were mixed with a convalescent serum (diluted 1:5) from a volunteer previously ill with Hawaii-induced disease, the particle was observed to be in aggregates, and it appeared to be covered with antibody. It was necessary to concentrate stool filtrates used in these studies in order to consistently visualize enough particles to assess antibody level in serum. Paired sera were then tested using the technique previously used with the Norwalk particle. Using acute and convalescent sera from the original case of Hawaii disease, a significant antibody response was observed. Subsequently, paired sera from 10 volunteers ill with Hawaii-induced gastroenteritis were tested and 7 of the 10 had a significant antibody response. Paired sera from persons ill with hepatitis A or Norwalk-induced gastroenteritis did not demonstrate serum antibody increases to the Hawaii particle. Further studies are now underway to confirm these findings and to characterize the Hawaii particle using biophysical methods.

We recently expanded our gastroenteritis studies which had been limited to outbreaks in schools, institutions, the military, and families, to include infants and young children admitted to the Children's Hospital of the District of Columbia with acute gastroenteritis. Filtrates of stools collected during January, February and March, 1974 from 21 infants and children, 2 to 20 months of age, who had gastroenteritis with vomiting and/or diarrhea severe enough to warrant hospitalization were studied for the presence of viral agents by immune electron microscopy (IEM) and in some instances by conventional electron microscopy as well. Virus particles with characteristic reovirus-like morphology were visualized in stool filtrates from 13 (62%) of the 21 patients. The particles were so distinct in morphology and in most instances so numerous that the 19 virus-containing filtrates examined by both conventional and immune electron microscopy were positive by both methods. The particles from the different patients appeared to be similar morphologically and had a definite capsomere structure. They resembled the reoviruses or orbiviruses morphologically. The diameter varied from approximately 67 to 73 nm with an average of approximately 70 nm. Many of the particles appeared to have a definite double shelled capsid; "empty" particles were seen occasionally. We also examined stools from 13 controls who included infants and children admitted to the hospital during February and March, 1974, for respiratory disease, and others returning for well baby care and we were unable to detect reovirus-like particles in these control stool filtrates.

We used a stool filtrate which had readily detectable particles to examine acute and convalescent sera from the gastroenteritis patients for antibody to this reovirus-like agent by IEM. Paired sera from 4 patients were examined by

this technique for antibody to the homologous agent present in stool; each individual demonstrated serologic evidence of infection to the reovirus-like agent. We also tested paired sera from each of these individuals and from an additional patient with gastroenteritis from whom a stool was not obtained for antibody to the virus present in the stool of one patient. Each of the five infants had serologic (IEM) evidence of infection with this reovirus-like agent indicating that the agents which infected these patients were either identical antigenically or very closely related.

We also were able to develop a complement-fixation test for the reovirus-like agent by utilizing as CF antigen 2% stool filtrate from one of the patients. In this test system using 32-64 units of antigen we not only confirmed the above described serologic (IEM) evidence of infection in 5 patients but also found that the remaining 5 reovirus-like positive gastroenteritis patients with paired sera had serologic (CF) evidence of infection. The varying dates of serum acquisition and differing residences of the patients makes it most unlikely that a single adventitious agent was responsible for these infections. It is noteworthy that paired sera were also available from 4 infants and children whose filtrates were negative for the reovirus-like agent and each failed to develop a significant seroresponse to the CF antigen, In addition, we tested paired sera from 4 volunteers who developed illness following challenge with the Norwalk agent and a significant increase in IEM antibody to the Norwalk agent during convalescence; none of the 4 volunteers developed a seroresponse to the reovirus-like CF antigen. Further evidence of specificity was provided by our failure to detect a CF antibody response in paired sera from volunteers infected with non-bacterial gastroenteritis agents derived from 6 separate family or institutional outbreaks. In addition, CF tests with paired sera from 10 other community-wide, institutional, or family outbreaks of gastroenteritis failed to demonstrate a relationship to the reovirus-like agent. We also were able to demonstrate that the reovirus-like antigen from patient D was distinct from reovirus types 1, 2 and 3 and from all orbiviruses for which antisera were available.

We have observed a reovirus-like agent in stools of infants and children with gastroenteritis and have for the first time demonstrated serologic evidence of infection with this agent, the latter accomplished by both IEM and complement fixation. These findings along with the absence of such particles in stools obtained from children without gastroenteritis suggests that this reovirus-like agent was the etiologic agent of the gastroenteritis observed in a majority of the hospitalized children. We have also demonstrated that the reovirus-like agent is antigenically related to the EDIM murine diarrhea virus and the Nebraska calf diarrhea virus (NCDV). In addition, development of a CF test for antibody to this reovirus-like antigen should greatly facilitate laboratory and seroepidemiologic studies of the agent. Preliminary seroepidemiologic surveys in adults from many parts of the U.S. reveal that CF antibody to the reovirus-like antigen is quite common in serum from adults indicating that this agent or a related agent is relatively ubiquitous. It is likely that this reovirus-like agent will emerge as a major etiologic agent in infantile diarrhea.

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Preliminary evidence was obtained that the reovirus-like agent of sporadic infantile diarrhea was grown in organ cultures of human fetal intestine. Cytoplasmic fluorescent staining was seen in the epithelial cells which lined the intestinal villi of inoculated organ cultures when convalescent serum containing CF antibody was used in the indirect fluorescent antibody. Acute phase serum, which lacked CF antibody, did not stain these areas. Each of the patients who had reovirus-like particles in their stools and who developed a serum CF antibody response to the agent developed a similar serum antibody response when tested by the indirect immunofluorescent technique. (Kapikian, Wyatt, Thornhill, Kalica and Chanock).

Hepatitis:

1973-74 was a banner year for hepatitis virus research in LID. Despite significant progress in recent years in the study of hepatitis B, little progress has been made in our understanding of hepatitis A because neither the virus nor infection caused by it could be identified. However, this year we detected a virus-like particulate antigen in the stool of patients acutely ill with hepatitis A and demonstrated its temporal and serologic relationship to hepatitis A infection. Based upon these findings it appears that the 27 nm particle is the hepatitis A virus. The antigen was detected, using the technique of immune electron microscopy, in stool samples collected only during the acute phase of infection. Using these particles as antigen, the development of antibody could be demonstrated during convalescence from hepatitis A infection. An antibody response to hepatitis A antigen was demonstrated with paired serum samples obtained from patients involved in 5 separate, naturally occurring outbreaks of hepatitis A as well as from volunteers involved in two studies of experimentally induced infection. The antigen and its antibody were found to be serologically distinct from hepatitis B antigen-antibody systems as well as from the morphologically similar virus-like antigen associated with Norwalk acute, non-bacterial gastroenteritis.

As part of a collaborative study of hepatitis A jointly funded by NIAID, FDA and CDC, marmosets were inoculated with hepatitis A virus. Tests of paired sera from these marmosets for development of antibody to hepatitis A antigen revealed an excellent correlation between clinical evidence of hepatitis and seroconversion; serologic tests were as sensitive as histologic and biochemical tests for detecting infection.

The hepatitis A antigen was found to have a buoyant density in cesium chloride of approximately 1.4 and a sedimentation coefficient in sucrose consistent with its size of 27 nm. These findings plus those obtained by others, using the marmoset model for characterization of the hepatitis A agent, suggests that this virus may be a parvovirus. More definitive classification must await the determination of the type of nucleic acid it contains.

Seronegative infant chimpanzees, obtained from a breeding colony jointly funded by NIAID, FDA, and CDC were inoculated with a stool filtrate prepared from stools collected during the acute phase of experimentally acquired hepatitis A infection. Both chimpanzees administered the stool filtrate developed hepatitis A as evidenced by symptoms, elevated transaminase levels, detection of hepatitis A antigen in stool samples collected during the acute phase of infection, and development of antibody to hepatitis A antigen during convales-

cence. In contrast, the two chimpanzees inoculated with control stool filtrates remained normal. Subsequently, these latter two animals developed evidence of hepatitis A infection following casual contact with the former two animals.

Surgical patients who were followed longitudinally and who developed post-transfusion hepatitis were carefully studied for evidence of infection with hepatitis B virus, cytomeglovirus or Epstein-Barr virus. Only half of the patients had evidence of recent cytomeglovirus infection. However, a comparable proportion of patients with type B hepatitis or no hepatitis at all had evidence of cytomegló infection. The etiologic relationship of this virus to post-transfusion hepatitis is therefore difficult to determine but is probably not significant. None of the patients had evidence of recent Epstein-Barr virus infection. Because it has been assumed that much non-B post-transfusion hepatitis is caused by hepatitis A virus, paired sera from non-B post-transfusion hepatitis patients were tested for development of antibody to hepatitis A antigen. None developed such antibody, and approximately half of those tested had evidence of prior infection with hepatitis A virus. These observations indicate that not all hepatitis is caused by hepatitis A and B viruses. We plan an intensive search for the hypothetical "hepatitis C virus" during the next year.

Advances were also made in the study of hepatitis B virus infection. A micro solid-phase radioimmunoassay specific for the core of the Dane particle (thought to be the hepatitis B virion) was developed. Radioimmunoassay exhibited a high degree of sensitivity and specificity. In a collaborative study with Drs. Kaplan, Greenman and Robinson of Stanford and Dr. Gerin of the MAN Laboratory, the micro solid-phase radioimmunoassay for core antigen was instrumental in localizing viral DNA polymerase activity to the core of the Dane particle, thus strengthening the case for the viral nature of the Dane particle. (Purcell, Feinstone, Kapikian, Gold, Moritsugu,Peterson).

Mycoplasma pneumoniae:

Last year we described the production and characterization of a series of temperature sensitive (ts) mutants of M. pneumoniae, the etiologic agent of cold agglutinin positive pneumonia. Mutants were selected which were restricted in growth on agar at 36°C, 37°C, or 38°C. At these temperature the wild type organism grew without restriction. Mutants of the ts class were sought with the expectation that their temperature sensitive defects would restrict growth in vivo at the temperature of the lower respiratory tract (37°C), whereas replication would not be seriously impaired in the cooler environment of the upper respiratory passages (32°-34°C). Mutants which were completely stable genetically in broth medium and in vivo in the hamster were selected for further study. These mutants were found to be attenuated for the hamster and to stimulate significant resistance to subsequent challenge with wild type organisms. The first mutant to be tested in man was ts-640. This mutant proved to be attenuated, however, it retained sufficient residual virulance to disqualify it as a candidate vaccine strain. Nonetheless, the results of the study were encouraging since ts-640 induced a significant antibody response in the volunteers and the mutant was genetically stable.

The next mutant to be tested in man was ts-H43 which was more temperature sensitive than the ts-640 mutant. Each of the 16 men given the mutant by the intranasal route became infected. Thirteen of the volunteers shed M. pneumoniae, while each of the men developed a significant rise in serum antibody as measured by CF, MI and/or MCT. None of the 78 isolates recovered from the volunteers produced colonies on agar at the restrictive temperature of 38°C, whereas colony formation was efficient at the permissive temperature of 32°C. A more stringent test for genetic alteration of the ts mutant was performed with the last isolate recovered from each infected volunteer. The last isolates were tested for efficiency of colony formation at both 37°C and 38°C in water baths which did not vary in temperature more than 0.05°C. None of the last isolates produced colonies at either 37°C or 38°C. These findings suggest that the ts-H43 mutant did not undergo a change in its ts phenotype during growth in man.

Because of the apparent importance of local immune mechanisms in resistance to M. pneumoniae disease we studied the secretory IgA antibody response of volunteers infected with the ts-H43 mutant. Thirteen of the 16 men were able to produce serial sputum samples. Eleven of these men (84%) developed a significant (i.e., 3-fold or greater) increase in M. pneumoniae IgA antibody as measured by radioimmunoprecipitation using ^{3}H-oleic and ^{3}H-palmitic acid labelled organisms. The secretory antibody responses to ts-H43 appeared to occur as frequently and to be of the same magnitude as the responses of the volunteers who were infected with a partially attenuated wild type (ts+) suspension of M. pneumoniae. In addition, the secretory antibody responses of the ts-H43 infected volunteers were similar in frequency and magnitude to those described for a larger group of men infected with virulent wild type M. pneumoniae.

None of the men infected with the ts-H43 mutant developed any signs or symptoms of illness during the 28 day period of intensive medical surveillance following administration of the inoculum. There were no elevations of temperature nor did any of the men develop any signs or symptoms involving the respiratory tract.

In summary, in the present study each of 16 volunteers underwent a completely silent infection with mutant ts-H43. Infection of the respiratory tract was extensive enough, however, to stimulate a moderate local secretory antibody response as well as a systemic antibody response. These observations suggest that the ts-H43 organism had achieved the desired balance between attenuation (defectiveness) and antigenicity. The development of a respectable local antibody response was particularly encouraging because of the mounting evidence that local immunolgoical factors in the respiratory tract play a major role in protecting the host against M. pneumoniae disease.

Preliminary observations from a challenge of ts-H43 vaccinees with partially attenuated wild type (ts+) M. pneumoniae suggested that the mutant had induced resistance to infection. In the challenge study resistance to illness could not be evaluated, however, infection with wild type (ts+) organisms appeared to be suppressed in men previously given the ts-H43 mutant.

In practical terms the results of the first volunteer studies with M. pneumoniae mutant ts-H43 are sufficiently encouraging to warrant cautious expansion of trials with this mutant in larger numbers of volunteers. Furthermore, while several attempts to use temperature sensitive mutants of respiratory viruses for vaccine purposes have yielded encouraging results, the present effort represents the first application of this technique to free living organisms. The promising results presented here may offer a new approach toward the control of other bacterial respiratory pathogens. (Greenberg, Helms, Chanock).

Respiratory Syncytial Virus:

Previous work indicated that the 7 existing, stable, temperature sensitive mutants of RSV could be placed in three separate complementation groups. The first group included ts-1, 3, 4, 5 and 6, mutants produced from the wild type A2 strain by treatment with the chemical mutagen 5-FU. The second group included a single mutant, ts-7, produced by ethylmethanesulfonate. The third group contained ts-2 a 5-FU-induced mutant that exhibited altered plaque morphology and considerable leakiness. Further studies of these mutants indicated that the ts-7 lesion probably affected an earlier function than those affected in the other mutants. Our current work has further clarified the physiology of these mutants by elucidating patterns of virus-induced RNA production in mutant-infected cells grown at both permissive temperature (32°C) and restrictive temperature (39°C). Attempts were made to examine the possibility that the mutant RNA polymerases were temperature sensitive. The results of these studies suggested that the function of RNA polymerase at restrictive temperature was not affected by the genetic lesion (or lesions) in any of the ts mutants.

Further experiments were then performed to examine whether some step prior to RNA synthesis was affected in each of the mutants. In these experiments it was found that ts-2, the leaky mutant, incorporated ^{3}H-uridine into RNA with equal facility after incubation at either permissive or restrictive temperature. These results indicated that all the mutants except ts-2 were defective in some function occurring prior to RNA synthesis in the viral growth cycle. This finding seemed somewhat paradoxical since each mutant (with the exception of ts-4) had been shown to be able to synthesize some viral specific protein when grown at restrictive temperature. Thus, protein synthesis occurred at restrictive temperature in the absence of RNA synthesis. Workers in other laboratories have noted similar findings in the case of temperature sensitive mutants of vesicular stomatitis virus. Possibly the paradox can be explained by assuming that different enzymes or conditions are required for the transcriptive and replicative aspects of viral RNA synthesis. At restrictive temperature, the mutants appear to be incapable of RNA replication but may be capable of initiating a small amount of RNA transcription resulting in the formation of messenger RNA in sufficient quantities to direct detectable amounts of protein synthesis. This amount of RNA might not be sufficient to be detected by the methods employed. The methods employed might be sensitive enough to detect RNA synthesis only when larger quantities of messenger RNA were made or when both RNA transcription and RNA replication were occurring normally.

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The effects of a glucose analogue, 2-deoxy D-glucose (2-dG) on the physiology of RSV and of parainfluenza virus type 3 (para 3) and of measles virus were examined in detail since several other enveloped viruses were recently found to be readily inhibited by this drug which interferes with glycosalation of envelope glycoproteins. 2-dG in a concentration of 1 mM or greater depressed replication of RSV, para 3, and measles viruses and dramatically prevented the production of cytopathic effects by these viruses. 2-dG exerted its effects in the latter half of the viral growth cycle, and removal of the agent at midcyle indicated that the action of the drug was completely reversible. 2-dG led to a cessation of viral growth within 2 hours. Viral growth proceeded at an accelerated pace following removal of the drug indicating that 2-dG blocked a specifiv step in virus maturation and that earlier functions occurred normally, allowing for accelerated growth when the block was removed. A further indication that 2-dG allowed at least some viral function to proceed normally was seen in immunofluorescent studies where the production of viral antigens (as indicated by indirect immunofluorescence) was found to be unimpaired in the presence of 2-dG at a concentration as high as 10 mM. Electron microscopic (EM) studies were carried out using the RSV ts-2 mutant which produces abundant viral structures. In the presence of 2-dG, ts-2 was inhibited to the same extent as wild type RSV. When 2-dG was added to the medium, the number of virions released from infected cells was markedly reduced. Moreover, those virions which were released seemed to be denuded of surface spike projections. As these spikes have been associated with glycoproteins in the paramyxovirus group, this result was regarded as evidence that 2-dG interferes with glycopeptide metabolism in RSV as it has been shown with other enveloped viruses.

Because of its marked effect in vitro 2-dG was studied for a possible antiviral effect in vivo. Thus far, we have been unable to alter the course of RSV or para 3 infection in hamsters or influenza A virus infection in mice. These negative results were obtained despite the fact that inhibitory blood concentrations of 2-dG were obtained by means of parental injection of the drug. It was considered likely that the drug did not reach the actual site of viral infection, viz., the respiratory epithelium. Delivery of 2-dG by aerosol, however, did not result in inhibition of virus growth in the lungs. (Hodes, Schnitzer, Kalica, Chanock).

Influenza Viruses:

During the past year considerable progress was made in elucidating the nature of the genetic lesions present in the temperature sensitive (ts) mutants of influenza A virus produced in this laboratory. The relationship of 13 mutants to each other was investigated in some detail. Four of the viruses were recombinants produced by mixed infection of influenza A (H3N2) 1968 wild type and influenza A (H2N2) ts-1 or ts-2 mutant. The other 9 mutants were produced by direct mutagenization of influenza A (H3N2) 1968 virus. We were particularly interested in ordering these viruses in terms of genetic relatedness since one of the purposes of this project was to derive mutants which had a single lesion (or cluster of lesions) on only one of the discrete RNA segments of the viral genome. Further it was our intention to produce single mutants with lesions specific for each of the genes of the influenza A virus. Such a set of stable, single lesion mutants should prove invaluable as genetic probes for characterization of the lesion or lesions transferred from an older ts

vaccine strain to a recently emerged, epidemic strain possessing new surface glycoprotein antigens. For example when the forthcoming influenza A (H4N3) (or H4N2) virus emerges we will transfer the ts-1[E] lesions into the new virus. At that time it will be necessary to authenticate the successful transfer of the ts-1[E] lesions to the new epidemic virus by crossing the H4N3 (or H4N2) ts recombinant with its ts-1[E] parent (i.e., backcross), the mutants containing the 2 single ts-1[E] lesions and finally the other single ts mutants. Availability of single lesion ts mutants should also allow us to define the relationship between site of genetic change in the genome and attenuation for experimental animals and man.

Pairwise analysis of the 13 mutants was performed by the monolayer culture complementation-recombination technique. In this test various pairs of mutants were mixed, incubated overnight at 4°C and then tested for their capacity to produce plaques on monolayer cultures at the restrictive temperature of 39°C. None of the ts mutants alone were able to produce a significant number of plaques at 39°C, whereas certain pairs of mutants produced plaques at the restrictive temperature with high efficiency. When plaques developed at 39°C following mixed infection the 2 viruses were considered to possess distinct, non-overlapping, genetic lesions; presumably each virus provided for its partner in the cross the gene product (or products) which was defective in the other. This type of complementation was followed by reassortment of viral genes and the production of wild type virions; this was confirmed by the finding that 90% of the progeny of 200 plaques produced at 39°C by mixed infection with various pairs of ts mutants exhibited the wild phenotype.

Seven of the 13 mutants or recombinants studied appeared to possess single, distinct ts lesions. Tentatively we have assigned these viruses to 7 complementation groups. This number of complementation groups is not exhorbitant since the influenza A virus genome is thought to contain 7 to 10 discrete pieces of RNA. Some of the mutants exhibited ambiguous behavior in the complementation-recombinant test and thus cannot be adjudged as single or multiple mutants or assigned to the tentative complementation groups.

The ts-1[E] recombinant of influenza A/Hong Kong/1968 (H3N2) was shown in previous studies to possess the properties desired of a live vaccine virus, i.e., infectivity for man, attenuation and non-transmissibility. During the past year the ts-1[E] virus was characterized in greater detail by transferring the ts lesion (or lesions) to other influenza A viruses and studying (a) the manner in which the ts lesion segregated and the genetic relationship of the various ts recombinants to each other; (b) the segregation of ts lesions with respect to surface glycoproteins; and (c) the relationship of the segregated ts lesions to temperature sensitivity, growth in the lungs of hamsters and virulence for man.

Hong Kong (H3N2) ts-1[E] virus was recombined with wild type influenza A/Marton/1943 (HON1) for the following reasons: (a) HON1 virus differs from the H3N2 virus in both surface glycoproteins thereby permitting exploration of the relation of the ts-1[E] lesions to the 2 surface antigens; (b) young adults born after 1947 lack antibody for the HO antigen and therefore could serve as seronegative volunteers in studies designed to evaluate the attenuation of HO ts recombinants. In this sense the production and evaluation of HO ts-1[E]

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recombinants represents a model for the attenuation of the next pandemic virus (H4N3 or H4N2). Following mixed infection of bovine kidney cultures with Hong Kong (H3N2) ts-1[E] virus and Marton (HON1) virus 300 clones with the HO surface antigen were examined and 10% were found to be ts. Fifteen of the ts clones were studied in greater detail. From this analysis the following information emerged: (a) two of the HO ts-1[E] recombinants appeared to contain distinct lesions and these viruses (R1 and R8) did not undergo complementation-recombination with their H3N2 ts-1[E] parent. This suggests that the H3N2 ts-1[E] parent contained 2 distinct genetic lesions; (b) the majority of the HO ts-1[E] recombinants appeared to have received both (R1 and R8) lesions; i.e., they were double mutants; (c) one of the putative recombinants (R9) underwent interaction with R1, R8, the other double recombinants and the H3N2 ts-1[E] parent. This R9 virus probably represents the isolation of a spontaneous mutant and re-emphasizes one of the dangers inherent in genetic manipulations with influenza A viruses; (d) the 2 ts-1[E] lesions (R1 and R8) segregated independently of the neuraminidase antigen. Previously it had been shown (and again confirmed in the present studies) that neither of the ts-1[E] lesions were located on the gene which codes for the hemagglutinin.

The observation that the ts-1[E] virus lesions are not related to either surface antigen is of some importance because it means that one can produce temperature sensitive ts-1[E] recombinant vaccines with any desired combination of hemagglutinin and neuraminidase antigens as new antigenic variants emerge and assume public health importance.

Following the emergence of the 1972 London antigenic variant of influenza A (H3N2), which differed appreciably from the 1968 Hong Kong virus, we produced ts-1[E] recombinants of the 1972 virus. Recombinants were selected following mixed infection with wild type Udorn 1972 virus (identical to the 1972 London prototype) and Hong Kong ts-1[E] virus. Since both viruses were within the same H3N2 subtype it was necessary to treat the progeny of the mixed infection with antiserum monospecific for the 1968 H3 antigen at a dilution which did not neutralize virus bearing the 1972 H3 antigen. Of 42 plaques analyzed 29% were temperature sensitive and the virus progeny bore the 1972 H3 antigen. Seven of these ts recombinants were studied in greater detail and it was found that 4 had a 39°C shutoff temperature, while 3 were restricted at 38°C like the 1968 Hong Kong ts-1E] parent. None of the 7 recombinants recombined with each other or with the 1968 H3N2 ts-1[E] parent or with R11 (an HO ts-1[E] recombinant carrying both ts-1[E] lesions). However, the 39°C shutoff mutants behaved like the HO R1 recombinant and recombined with the HO R8 recombinant but not R1 or R11. In contrast the 3 38°C shutoff recombinants received both ts-1[E] lesions, while only one lesion (R1) was transferred to the 4 39°C mutants.

In the hamster growth of the 38°C shutoff mutants in the lungs was reduced at least 300-fold compared to wild type virus, while the 39°C mutants exhibited an intermediate pattern of virus replication in the lungs. Growth of the ts recombinants was more restricted in the lungs than in the nasal turbinates. This is not surprising since the temperature of the hamster's lungs is approximately 37°C, whereas the turbinates are considerably cooler (32-34°C). This type of differential suppression of growth of ts viruses in lungs versus turbinates was also seen with the 1968 Hong Kong ts mutants and the HO ts-1[E] recombinants and appears to be a general phenomenon.

Reversion to wild type virus was seen occasionally on direct assay of the lungs of hamsters infected with Hong Kong ts-1[E] virus but not with a 38°C Udorn ts-1[E] recombinant. However, when tissue culture isolates were examined revertants were detected with greater frequency, but the majority of virus in the lung retained its ts phenotype. Reversion was seen considerably less frequently in nasal turbinate grown virus, probably because the lower ambient temperature (32-34°C) in the nose exerted less selective pressure than the higher temperature of the lungs (37°C).

Two of the Udorn ts-1[E] recombinants were evaluated in seronegative volunteers--clone 13 with a 39°C shutoff temperature and clone 16 with a 38°C shutoff temperature. Fifteen of the 19 men given clone 13 virus were infected and 4 developed mild but definite febrile respiratory illness. This clone is presumed to contain only one of the 2 ts-1[E] lesions. Ten of the 16 men given the clone 16 virus were infected but none of the volunteers became ill. Clone 16 is presumed to contain both ts-1[E] lesions. Thus, the recombinant which contained only one of the 2 ts-1[E] lesions appeared to be less temperature sensitive, less restricted in vivo in the hamster and less attenuated in man than the putative double recombinant (which contained both ts-1[E] lesions). At this time our experience with the Udorn ts-1[E] recombinants conforms to our working hypothesis that the defined ts-1[E] lesions are associated with a predictable level of attenuation when transferred into a wild type influenza A virus bearing new surface antigens. (Murphy, Richman, Stewart, Chanock).

Mycoplasma:

The role of mycoplasma toxins and other membrane or cell-associated factors in these organisms that may account for their attachment and toxicity to host tissue cells are under intensive investigation. There are increasing reports of neurological disease associated with M. pneumoniae infections in man and studies on the most well known neurotoxic mycoplasma (M. neurolyticum) can provide basic information on biological factors required for the elaboration of toxin and the chemical nature of this toxin. Our finding of a neurotoxic effect in primates reported here is the first evidence that the toxin of M. neurolyticum might be involved in neurologic disease in hosts other than rodents. Primates also might be the logical host to assess the role of other mycoplasmas of human origin in neurologic disease.

Carbohydrates, including glycoproteins, glycolipids and polysaccharides appear to represent only a minor component of the total membrane constituents of mycoplasmas. However, there is increasing evidence that several of these substances play a major role in the serological activity of some pathogenic mycoplasmas and there is speculation that carbohydrate-containing compounds are involved in the adsorption of mycoplasmas to host cells. The indirect evidence obtained this year suggesting the presence of exposed carbohydrates on the surface of mycoplasma membranes adds another dimension to the structure of the mycoplasma membrane. Further investigation on the role of these compounds in the interaction of mycoplasmas and tissue cells is clearly indicated. (Tully).

Serial No. NIAID 66A
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Epidemiologic Studies of Infections and Illness, and Their Control in Infants and Children

Previous Serial Number: Same

Principal Investigator: Dr. Albert Z. Kapikian

Other Investigator: Dr. Robert M. Chanock

Cooperating Units: Mr. W. Lee Cline, Serology Unit, LID, NIAID

Contract Support: None

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	10/12			10/12
Professional:	< 1/12			< 1/12
Other:	10/12			10/12

Project Description:

This was a long term, intensive study of the illness and microbial experience of infants and children at Junior Village, a District of Columbia Welfare Institution. This study began in July of 1955, and the field portion of the study was discontinued on May 21, 1969.

Basic Objectives:

The general objective was to maintain under observation a population group suitable for epidemiologic study of the occurrence of infections and disease and, in addition, to study host-parasite-disease relationships as they occur naturally and as they could be altered by vaccines and/or chemo-prophylactic agents. A concerted effort was made to (a) find new microbiologic agents which cause disease; (b) find methods of identification of these agents; (c) define modes of spread of infectious agents; and (d) find methods for prevention and treatment of acute illnesses, particularly respiratory illnesses.

Methods Employed and Patient Material:

The field portion of this study was discontinued on May 21, 1969. The epidemiologic setting was described in detail in previous annual reports.

Currently, most of the original throat swab specimens from the study are stored at -70°C for the study of coronaviruses when suitable methods for their propagation become available; in addition, anal swab specimens are stored at -20°C for the study of gastroenteritis agents when suitable methods for their propagation are found and for electron microscopic studies as well.

Major Findings:

Epidemiology of Rhinovirus Infection and Characterization of Rhinoviruses - As we described in last years' annual report, most respiratory viruses have been studied extensively at Junior Village. However, one major group which has not been studied extensively has been the rhinoviruses since antisera were not available for many serotypes and as a result typing of strains on a limited basis was generally unsuccessful. However, with the completion of the second phase of the rhinovirus collaborative program and the availability of antisera to all 89 serotypes and the one subtype, efforts will be made to study the epidemiology of the numerous rhinovirus serotypes at Junior Village. Thus far, acid lability tests have been carried out on 344 viral isolates from 222 residents and 122 viruses have been found to be acid labile. No additional testing was carried out since the previous report.

An Outbreak of Infectious Lymphocytosis - As described in last years and previous annual reports, this outbreak occurred in a four month period when at least 22 cases of acute infectious lymphocytosis were discovered. The leukocyte counts varied from 20,000 to 94,000 per mm. with a relative lymphocytosis on the average of approximately 80%. Routine viral, bacterial, fungal, and parasitic studies were performed on specimens obtained during the outbreak and no definitive etiologic agent was found. Renewed attempts will be made to isolate or detect an etiologic agent by using newer techniques such as organ cultures, experimental cell cultures, and immune electron microscopy. The occurrence of EB virus infection in 10 children with infectious lymphocytosis was studied using the immunofluorescent technique. These studies which were reported in a previous annual report revealed that none of 6 children without pre-existing antibody, and none of 4 with such antibody developed four-fold or greater EB virus antibody rises. In addition, paired sera from 9 of the 10 children were tested by the CF technique for evidence of infection with cytomegalovirus (CMV) and herpes simplex virus (HSV); no rises to CMV and one to HSV were detected. No studies on this outbreak were carried out in addition to those described in last years and previous annual reports.

Seroepidemiological Studies of Coronavirus Infections - In last year's and a previous annual report, we described the incidence of OC 43 and 229E virus infections as determined by serologic (CF) tests of paired sera obtained from children admitted to Junior Village between October 15 and December 15 during the years 1961 through 1968. This period had been selected in an attempt to bracket the "winter" seasons with a serum collected on admission to the cottage and a serum taken several months later since it had been

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shown that adult coronavirus infections were prevalent during both a "winter" and "winter-early spring" period. Since it was found that 23 (16%) of 148 children developed significant CF antibody rises to OC43 virus (in contrast, however, only 1 of 149 developed such a 229E virus antibody rise), these seroepidemiologic studies were extended to include sera obtained during all periods of the year. In addition, in these expanded studies, the sera are tested not only by the CF technique for 229E and OC43 virus antibody, but also by the HI technique for OC43 virus antibody. These seroepidemiologic studies will be continued and further analysis made in an attempt to determine the importance of coronaviruses in this population. Selected throat swab specimens will also be tested in HEI MA-177 cell cultures in an attempt to recover coronaviruses from those periods shown by serologic tests to have had a high incidence of coronavirus infections.

Significance to Biomedical Research and the Program of the Institute:

This project represents a systematic approach to determining the various etiologies of acute febrile illnesses. Since carefully gathered epidemiological data and clinical specimens are available, this project also offers an unusual opportunity for the study of the epidemiologic patterns of infections associated with newly discovered agents. We will attempt to study the role, if any, of the reovirus-like gastroenteritis agent in nonbacterial gastroenteritis among residents of Junior Village since both sera and anal swab specimens are available.

Proposed Course of Project:

The field portion of this study was discontinued on May 21, 1969, when it became apparent that vaccine studies could not be carried out in this population. Studies relating to this project will be continued as long as profitable.

Honors and Awards: None

Publications: None

Serial No. NIAID 66H
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Etiology of Acute Infectious Nonbacterial Gastroenteritis

Previous Serial Number: Same

Principal Investigators: Dr. Richard G. Wyatt
Dr. Albert Z. Kapikian
Dr. Thomas S. Thornhill
Mr. Anthony R. Kalica

Other Investigators: Dr. Robert M. Chanock

Cooperating Units: Dr. Raphael Dolin, (LCI, NIAID)
Dr. Robert H. Parrott, Dr. Hyun Wha Kim, Dr. Carl Brandt, Dr. William J. Rodriguez and Dr. Sydney Ross (Children's Hospital, Washington, D. C.)
Dr. John L. Gerin (MAN Laboratory)
Dr. Arnold Levy (NIAMDD, D&HD)
Dr. William Woodward and Dr. Richard B. Hornick (University of Maryland)
Mr. W. Lee Cline (Serology Unit, LID, NIAID)
Dr. Lewellys F. Barker (BOB)
Dr. Robert H. Purcell (LID)
Dr. S. B. Mohanty (University of Maryland)
Dr. William T. London (NIMDS, PRB)

Contract Support

Contract Project Number	Project Officer (NIAID)	Contractor	Contractor's Project Director	Annual Funding Level
NO1-AI-32521 Part B	Dr. Chanock	Flow Laboratories, Inc.	Dr. Gilden	$210,000
NO1-AI-32505	Dr. Chanock	University Hospital Boston, Mass.	Dr. N.R.Blacklow	$45,000

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	70/12			70/12
Professional:	30/12			30/12
Other:	40/12			40/12

Project Description

Objectives: (1) To search for viruses which may play an etiological role in the syndrome of acute infectious nonbacterial gastroenteritis of infants, children, and adults. (2) to cultivate (in vitro) the viral agent(s) of acute infectious nonbacterial gastroenteritis; (3) to study the biophysical, immunological, and epidemiological characteristics of such agent(s); (4) to reproduce the syndrome experimentally in volunteers for the purpose of a) studying the pathophysiological and immunological responses of the host, and b) assaying the infectivity of such viral agents.

Methods Employed

(1) Further clinical materials were received from additional recent outbreaks of acute infectious nonbacterial gastroenteritis: Camp Nicodemus, Carroll County, Maryland (courtesy of Dr. William Barker, NCDC);Passavant Hospital), Chicago, Ill. (courtesy of Dr. R. Kaslow, Northwestern University); William & Mary College (courtesy of Dr. Richard Cilly); Maryland Nursing Home (courtesy Dr. Young, Md. State Health Department); Bellevue Hospital, N.Y., N.Y. (courtesy Dr. R. M. Maulitz, NCDC-NYC, Department of Health; Washington, D.C. (courtesy Dr. D. Wallis, D.C.Health Department). Materials were also collected from infants and children hospitalized at D.C.Children's Hospital, Washington, D.C., as a part of a preliminary search for viral agents of gastroenteritis in that age group.

(2) Two percent stool filtrates were prepared by centrifugation and Millipore membrane filtration. Selected filtrates were further concentrated by Amicon ultrafiltration and purified by density gradient ultracentrifugation techniques.

(3) Using stool filtrate as a source of antigen and paired sera from naturally occurring and experimentally induced cases of acute infectious nonbacterial gastroenteritis serologic tests were performed utilizing immune electron microscopy, complement-fixation, and indirect fluorescent antibody staining techniques.

(4) Chimpanzees, rhesus monkeys, baboons and calves were administered orally a stool filtrate derived from the Norwalk, Ohio outbreak of nonbacterial gastroenteritis in an attempt to demonstrate an animal model for this disease.

(5) Further volunteer studies were carried out using stool filtrate preparations and human fetal intestinal organ culture-derived materials. Studies were done at the NIH Clinical Center; General Clinical Research Center, University Hospital, Boston, Mass.; and the Maryland House of Correction, Jessup, Maryland.

Major Findings:

I. Further Studies with the Norwalk Agent of Acute Infectious Nonbacterial Gastroenteritis -

(A) Summary of Previous Findings - Using immune electron microscopy a 27 nm particle was visualized in a diarrheal stool filtrate (8FIIa) from an individual whose disease had been experimentally induced by administration of a Norwalk-derived stool filtrate. It was possible to use this technique to assess levels of serum antibody to the particle and to detect antibody rises following both naturally occurring illness and illness induced by Norwalk-derived filtrates. Based on this and other evidence, it was postulated that the 27 nm particle was the etiologic agent of this particular outbreak of acute gastroenteritis. The Norwalk particle was subsequently banded by ultracentrifugation in cesium chloride, and the distribution of particles was determined by immune electron microscopy. It was found to have a buoyant density of 1.38-1.41 g/cm^3, and on the basis of this and other data, it was suggested that the 27 nm particle was parvovirus-like.

(B) Particle shedding - To further define the Norwalk particle as the etiologic agent of the Norwalk, Ohio outbreak, we attempted to visualize this particle in stools of volunteers experimentally infected with the Norwalk-derived stool filtrate. In addition we examined the relationship between the appearance of the Norwalk particle in stool during the course of experimental infection and the onset of symptomatic illness.

Stool filtrates were prepared as a 2% suspension in veal infusion broth and filtered as previously described. Immune electron microscopy was performed on each stool filtrate using an aliquot of a single 4-week convalescent antiserum. This antiserum was selected because it contained a high level of antibody and, when present, the Norwalk agent appeared as single particles or small aggregates heavily coated with antibody. These conditions not only allowed easy identification of the Norwalk agent but also more evenly distributed the Norwalk agent over the grid and allowed more reliable particle counting.

Stool specimens from 23 volunteers, who became clinically ill after inoculation with the Norwalk agent, were examined in this study. The Norwalk agent was visualized by immune electron microscopy in a specimen (or specimens) from 11 of the 23 volunteers.

A total of 77 stool filtrates were examined by immune electron microscopy from these 23 volunteers. The Norwalk agent was detected in 25 of 53 specimens collected during the first 72 hrs after onset of clinical illness. Two of 12 specimens obtained after 72 hrs were positive while none of 12 specimens obtained before the onset of illness were positive.

The presence of the Norwalk agent in stool filtrates shortly after the onset of clinical illness and the failure to find the Norwalk agent before the onset of clinical symptoms or after recovery from illness further suggests that it is the etiologic agent of acute gastroenteritis of the Norwalk, Ohio outbreak.

(C) Infection of Chimpanzees with Norwalk Agent - The 8FIIa stool filtrate containing the Norwalk agent was administered by stomach tube to chimpanzees in an attempt to induce experimental disease in the species. Significant post-challenge serum antibody rises to the 27 nm Norwalk virus-like particle were demonstrated in all animals, using immune electron microscopy. Overt clinical illness, characterized by diarrhea and/or vomiting, did not occur. Transmission of infection was attempted subsequently by feeding additional chimpanzees a stool filtrate prepared from one of the previously challenged animals. Development of an antibody response to the particle in chimpanzees given this passage filtrate suggested that viral replication had occurred in chimpanzees in the absence of definite clinical illness. This animal model should facilitate further investigations with viral agents of acute gastroenteric disease. Rhesus monkeys, baboons, and calves did not develop serum antibody responses when given the same filtrate orally.

(D) Biophysical purification of the Norwalk agent - Attempts were made to purify the Norwalk agent from stool specimens obtained from volunteers experimentally infected with Norwalk stool filtrates. Stool filtrates were prepared as previously described and then concentrated (10x) in an Amicon ultrafiltration system. These 10x concentrates were then purified both by rate sedimentation and isopycnic banding in cesium chloride. Norwalk particle containing fractions of 2, 3, and 4-step banded material were labeled with NaI^{125} using either chloramine-T or lactoperoxidase. Assessments of purity of the fraction were made by electron microscopy, UV absorption analysis, and polyacrylimide gel electrophoresis of NaI^{125} labeled specimens. The major problems encountered during these studies have been two-fold: (1) two and even three-step banded stool filtrates did not attain the purity necessary for specific labeling and (2) losses during the process of purification plus the low titer of Norwalk particle present in the original stool filtrates resulted in such a low concentration of particles after the fourth step purification that specific labeling was not possible. Present studies have involved immunochemical techniques to enhance purification without associated loss of particles.

In addition, during the course of determining shedding of the Norwalk agent we have examined many diarrheal stools and assessed particle concentration. When a high titered stool is found, we have prepared a large stool filtrate from this material and used the filtrate as a source of antigen.

(E) Serologic assay of Norwalk agent - At present immune electron microscopy is the only serologic test available for assay of Norwalk specific antibody. Complement fixation tests using Norwalk filtrates as an antigen source and either paired sera from experimentally infected volunteers or hyperimmunized guinea pig sera have not yielded specific results. Hemagglutination has been seen with Norwalk-derived filtrates but hemagglutination inhibition by convalescent Norwalk antiserum was not obtained.

In addition, hemagglutination was seen with stool filtrates derived from control stool specimens and the presence of a soluble hemagglutination is not due to the presence of the Norwalk particle.

Countercurrent immune electrophoresis using Norwalk stool filtrates as the antigen source and paired sera from experimentally infected volunteers or hyperimmunized guinea pigs has not been conclusive.

A double antibody precipitation radioimmunoassay using NaI^{125} labeled partially purified Norwalk containing material was attempted. The lack of specificity of this test probably reflects the lack of purification of the labeled antigen source. IgG fractions from paired sera of an experimentally infected volunteer were labeled with NaI^{125} and used in a solid phase radioimmunoassay system. Lack of specificity found thus far in this test probably reflects the low titer of Norwalk agent specific antibody present in these IgG fractions. Present studies involve purification of Norwalk specific antibody from these IgG fractions.

II. Further Studies with the Hawaii Agent of Acute Infectious Nonbacterial Gastroenteritis -

(A) Histopathological Findings - In collaboration with Dr. Raphael Dolin and Dr. Arnold Levy, the intestinal histopathology induced by the Hawaii agent was investigated. This study was carried out in a similar fashion to the previous investigation of the histopathology induced by the Norwalk agent. Jejunal biopsies were obtained from 7 normal volunteers (a) prior to, (b) 48 hrs. after, and (c) at least 2 weeks after oral administration of the Hawaii agent of nonbacterial gastroenteritis. Tissue obtained was processed for light microscopy, electron microscopy, adenyl cyclase analysis, fluorescent staining, and in vitro propagation of tissue explants. Four of the seven volunteers developed definite typical illness and one of the volunteers had probable illness; two volunteers developed no symptoms. The evaluation of tissue by microscopic techniques, thus far, reveals a reversible lesion of the jejunum characterized by blunting of villi and a mild mononuclear cell infiltrate, in addition to blunted microvilli, swollen mitochondria, and amorphous electron dense material in intercellular spaces. There were no detectable abnormalties of the biopsy tissue analyzed for adenyl cyclase, and no fluorescent stainable antigen could be demonstrated in acute phase biopsies using indirect immunofluorescent techniques.

(B) Immune Electron Microscopic Studies of Hawaii Outbreak-derived Filtrate

On examination of additional diarrheal stool filtrates from volunteers ill with Hawaii agent-induced gastroenteritis, a small entero-or parvovirus-like particle was identified. When the filtrates containing this particle were mixed with a convalescent serum diluted (1:5) from a volunteer previously ill with Hawaii-induced disease, the particle was observed to be in aggregates, and it appeared to be covered with antibody. A large 2% stool filtrate (21DIIi), using the stool with most particles, was made for further studies.

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It was necessary to concentrate the stool filtrate used in these studies using an Amicon ultrafiltration apparatus in order to consistently visualize enough particles to assess antibody level in serum. Paired sera were then tested using the technique as had previously been done with the Norwalk particle. Using acute and convalescent sera from the original case of Hawaii disease, a significant antibody response was observed. Subsequently paired sera from 10 volunteers ill with Hawaii-induced gastroenteritis were tested and 7 of the 10 had significant antibody responses. Three additional volunteers had a demonstrable level of antibody in the pre-serum which was comparable to that found in the convalescent serum. Paired sera from persons ill with hepatitis A or Norwalk-induced gastroenteritis did not demonstrate serum antibody increases to the Hawaii particle. Further studies are now underway to confirm these findings and to characterize the Hawaii particle using biophysical methods.

(C) Additional Organ Culture Studies with Hawaii Agent - Additional attempts were made to cultivate in vitro the Hawaii agent using human fetal intestinal organ cultures. It had appeared that 3 of 14 men who received 11th passage organ culture material had become ill, and an additional experiment was designed to investigate this further. The inoculum used in these studies (21-11) was carried two additional passages in intestinal organ culture, and the resulting tissue culture fluids (21-13) were administered orally to 4 volunteers. None of these volunteers developed any signs or symptoms of gastroenteritis. This would suggest that the Hawaii agent does not replicate consistently in intestinal organ culture, or perhaps that virus attenuation may have occurred.

III. Nonbacterial Gastroenteritis of Infants and Children -
(A) Detection of Reovirus-like Particles by Immune Electron Microscopy and Complement Fixation - We recently expanded our gastroenteritis studies which had been limited to outbreaks in schools, institutions, the military, and families, to include infants and young children admitted to the Children's Hospital of the District of Columbia with acute gastroenteritis. Prior to studies by Bishop, et al., Flewett et al., and Bortolussi, et al., attempts to find such agents in this age group had been almost completely unsuccessful.

Filtrates of stools collected during January, February and March, 1974 from 21 infants and children 2 to 20 months of age who had gastroenteritis with vomiting and/or diarrhea severe enough to warrant hospitalization were studied for the presence of viral agents by immune electron microscopy (IEM) and in some instances by conventional electron microscopy as well. Stool filtrates from 14 of the patients were tested using their convalescent serum. In 7 instances, convalescent serum was not available for use in the IEM test; stool filtrates from these 7 patients were examined using a convalescent serum from either of 2 patients (Ru and B). Virus particles with characteristic reovirus-like morphology were visualized in stool filtrates from 13 (62%) of the 21 patients. A total of 44 stools was collected from the 21 children and 26 were positive for the reovirus-like agent. The particles were so distinct in morphology and in most instances so numerous that of the

19 virus-containing filtrates examined by both conventional and immune electron microscopy each was positive by both methods. The particles present in the 26 positive stools appeared to be similar morphologically and had a definite capsomere structure. They resembled the reoviruses or orbiviruses morphologically. The diameter varied from approximately 67 to 73 nm with an average of approximately 70 nm. Many of the particles appeared to have a definite double shelled capsid; "empty" particles were seen occasionally.

We also examined stools from 14 controls who included infants and children admitted to the hospital during February and March, 1974, for respiratory disease, and others returning for well baby care and we were unable to detect reovirus-like particles in 13 of these control stool filtrates. Reovirus-like particles were detected in a filtrate derived from a stool from a 2 month old infant admitted to the hospital with pneumonia. However, this infant developed diarrhea shortly after admission to the hospital. The controls were not matched precisely in time with the diarrhea group.

We used a stool filtrate which had readily detectable particles to examine acute and convalescent sera from the gastroenteritis patients for antibody to this reovirus-like agent by IEM. 0.2 ml of a 1:5 dilution of uninactivated serum was mixed with 0.8 ml of the stool filtrate and incubated overnight (at least 17 hours) at room temperature and then prepared for electron microscopy. The relative concentration of antibody in each serum was estimated by scoring the amount of antibody coating the particle on a 0 to 4+ scale as determined by electron microscopy.

Paired sera from 4 patients were examined by this technique for antibody to the homologous agent present in stool; each individual demonstrated serologic evidence of infection to the reovirus-like agent. We also tested paired sera from each of these patients and from an additional patient with gastroenteritis from whom a stool was not obtained, for antibody to virus particle present in the stool filtrate of patient Ru. Each of the five infants had serologic (IEM) evidence of infection with the reovirus-like agent indicating that the agents which infected these patients were either identical antigenically or very closely related.

We also were able to develop a complement-fixation test for the reovirus-like agent by utilizing as CF antigen a 2% stool filtrate from one of the patients (D). In this test system using 32-64 units of antigen, we not only confirmed the above described serologic (IEM) evidence of infection in 5 patients but also found that the remaining 5 reovirus-like positive gastroenteritis patients with paired sera had serologic (CF) evidence of infection. The varying dates of serum acquisition and differing residences of the patients makes it most unlikely that a single adventitious agent was responsible for these infection. It is noteworthy that paired sera were also available from 4 infants and children whose filtrates were negative

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for reovirus-like agent and each failed to develop a significant seroresponse to the CF antigen. In addition, we tested paired sera from 4 volunteers who developed illness following challenge with the Norwalk agent and a significant increase in IEM antibody to the Norwalk agent during convalescence; none of the 4 volunteers developed a seroresponse to the reovirus-like CF antigen. However, each of the 4 men had pre-existing CF antibody.

Failure of Norwalk-infected volunteers to develop a seroresponse to the reovirus-like antigen as well as the lack of a significant response of the 4 diarrhea patients who did not have reovirus-like particles in their stools suggests that the responses of the particle-positive diarrhea patients were specific and not a manifestation of enteric disease in general. Further evidence of specificity was provided by our failure to detect a CF antibody response in paired sera from volunteers infected with nonbacterial gastroenteritis agents derived from 6 separate family or institutional outbreaks. In addition, CF tests with paired sera from 10 other community-wide, institutional, or family outbreaks of gastroenteritis failed to demonstrate a relationship to the reovirus-like agent.

We also were able to demonstrate that the reovirus-like antigen from patient D was distinct from reovirus types 1, 2, and 3. In addition, paired sera from 3 patients who developed seroresponses to the reovirus-like CF antigen were tested for CF antibody response to reovirus types 1, 2 and 3 and no seroresponse to any of the reoviruses was observed. We also found that the reovirus-like CF antigen failed to react with a 1:4 dilution of 3 different polyvalent immune mouse ascitic fluids which contained CF antibody to all described orbiviruses except that of African horse sickness. From these data, it appears that the reovirus-like agent is distinct antigenically from the known reoviruses and from all orbiviruses for which antisera are available.

Since the reovirus-like agent resembled morphologically the epizootic diarrhea of infant mice (EDIM) virus which causes a diarrheal disease in suckling mice, we utilized the CF test to determine if an antigenic relationship existed between these agents. The reovirus-like CF antigen reacted with EDIM immune mouse serum to high titer. In addition, paired sera from 9 of the infants and children and children who developed gastroenteritis and had serologic evidence of infection with the reovirus-like CF antigen were tested for a seroresponse to the EDIM virus by CF. Two individuals developed a significant CF antibody response to the EDIM antigen. From these studies it appears that the reovirus-like agent is related antigenically to the EDIM virus.

Since the reovirus-like agent also resembled morphologically the Nebraska calf diarrhea virus (NCDV) which causes a severe diarrheal disease in neonatal calves, we utilized both IEM and CF techniques to determine if an antigenic relationship existed between these agents. In studies by IEM,

we found that a 1:5 dilution of NCDV immune rabbit serum had significant antibody to the reovirus-like particle. In addition, 32 units of the reovirus-like CF antigen reacted with a 1:40 dilution of the NCDV immune rabbit serum. It appears from these studies that the reovirus-like agent is related to the NCDV also.

We have observed a reovirus-like agent in stools of infants and children with gastroenteritis and have for the first time demonstrated serologic eivdence of infection with this agent, the latter accomplished by both IEM and complement-fixation. These findings along with the absence of such particles in stools obtained from children without gastroenteritis suggests that this reovirus-like agent was the etiologic agent of the gastroenteritis observed in a majority of the hospitalized children. We have also demonstrated that the reovirus-like agent is antigenically related to the EDIM virus and the NCDV. In addition, development of a CF test for antibody to this reovirus-like antigen could greatly facilitate laboratory and seroepidemiologic studies of the agent. Preliminary seroepidemiologic surveys in adults from many parts of the U.S. reveals that CF antibody to the reovirus-like antigen is quite common in serum from adults indicating that this agent or a related agent is relatively ubiquitous. It is possible that this reovirus-like particle will emerge as a major etiologic agent of infantile diarrhea. Additional studies are in progress to determine if this reovirus-like agent belongs to the orbivirus or reovirus subgroup of the reoviridae family.

(B) Propagation of the Reovirus-like Agent from Stools of Infants and Children with Nonbacterial Gastroenteritis Using Human Fetal Intestinal Organ Culture - Using indirect immunofluorescent techniques, fluorescent stainable antigen has been demonstrated in human fetal intestinal organ cultures inoculated with stool filtrates derived from infants and children with nonbacterial gastroenteritis. Using convalescent serum, 4+ cytoplasmic staining has been observed primarily in epithelial cells whereas staining was not seen with acute phase serum. A serological response to the detectable antigen was demonstrated by staining with dilutions of acute and convalescent sera from these patients, and reovirus-like particles have been observed in an organ culture-derived tissue and fluid mixture. Previous attempts to cultivate this reovirus-like agent have been unsuccessful.

Significance to Biomedical Research and the Program of the Institute:

Acute infectious nonbacterial gastroenteritis is a common infectious disease which affects a broad segment of the population and was the second most common disease observed in a 10 year family study. Ultimate goals of prevention and therapy have been furthered by (1) the detection and preliminary characterization of three postulated etiologic agents of this disease, (2) the development of serologic assay systems by which the epidemiologic importance of the disease can be determined and (3) the ability to cultivate

in vitro one of these agents. Specifically, the progress in studying the reovirus-like agent(s) of gastroenteritis in infants and young children represents a significant step in understanding this disease of probable world-wide importance.

Proposed Course of the Project:

Intensive study of etiologic agent(s) from outbreaks of acute infectious nonbacterial gastroenteritis will continue, using known infectious materials obtained through volunteer studies. Clinical materials from new outbreaks and sporadic cases of gastroenteritis will be studied using laboratory methods which have proved successful in previous investigations. In vitro propagation of the agent(s) will be studied further using a variety of cell and organ culture systems. Further biophysical purification and characterization of these agents, development of more sensitive serologic assay systems, and methods for rapid diagnosis of this disease will be major goals. The study of nonhuman primates and other species of animals as models for acute infectious nonbacterial gastroenteritis will be pursued.

Honors and Awards:

Invited speaker at Gustav Stern Symposium on Perspectives in Virology IX in New York, New York. Presentation entitled "Detection by immune microscopy of fastidious agents associated with respiratory illness, nonbacterial gastroenteritis, and hepatitis A". (Dr. Kapikian) [February 4, 1973].

Invited speaker at Workshop on Parvoviruses at NIH, Bethesda, Maryland. Presentation entitled "Visualization by immune electron microscopy of a parvovirus-like agent associated with acute infectious nonbacterial gastroenteritis in humans". (Dr. Kapikian) [February 12, 1974].

PUBLICATIONS:

Agus, S.G., Dolin, R., Wyatt, R.G., Tousimis, A.J. and Northrup, R.S.: Acute Infectious Nonbacterial Gastroenteritis: Intestinal Hidtopathology. Histologic and Enzymatic Alterations During Illness Produced by the Norwalk Agent in Man. Ann. Int. Med. 79: 18-25, 1973.

Buscho, R.F., Wyatt, R.G., Dolin, R., Blacklown, N.R., DuPont, H.L., and Chanock, R.M.: Recurrent Institutional Outbreaks of Acute Infectious Nonbacterial Gastroenteritis: Epidemiology and Etiology. Amer. J. of Epid. 98: 192-198, 1973.

Wyatt, R.G., Dolin, R., Blacklow, N.R., DuPont, H.L., Buscho, R.F., Thornhill, T.S., Kapikian, A.Z., and Chanock, R.M.: Comparison of Three Agents of Acute Infectious Nonbacterial Gastroenteritis. J. Inf. Dis. (in press) 1974.

Serial No. NIAID 66H

Wyatt, R.G., Kalica, A.R., and Dolin, R.: Ultrastructural Study of Viral Replication in Human Fetal Intestinal Organ Culture by Cross-Challenge in Volunteers. Archiv. fur die gesamte Virusforschung, 40: 341-349, 1973

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Serial No. NIAID 69A
1. Infectious Diseases
2. Respiratory Viruses
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Study of Non-bacterial Respiratory Disease
A. Children

Previous Serial No.: Same

Principal Investigator: Dr. Robert M. Chanock

Other Investigators: Dr. David S. Hodes
Dr. Albert Z. Kapikian

Cooperating Units: Dr. R.H. Parrott, Children's Hospital Research Foundation of Washington, D.C.

Contract Support:

Contract Number	Project Off. (NIAID)	Contractor	Contractor's Proj. Dir.	Annual Funding Level
NO1 AI 32521	Dr. Chanock	Flow Labs.	Dr. Gilden	$ 430,000
NO1 AI 12091	Dr. Chanock	Children's Hosp. D.C.	Dr. Parrott	135,792

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	24/12			24/12
Professional:	6/12			6/12
Other:	18/12			18/12

Project Description

Objectives: (1) To search for new agents responsible for respiratory disease in infants and children; (2) to delineate the epidemiology and pathogenesis of viruses which have been recently associated with respiratory disease; (3) to define the major determinants of host resistance to infection and illness; (4) to continue the surveillance of viruses previously established as playing a role in respiratory disease, i.e., the epidemiology and contribution to overall respiratory disease experience during infancy and childhood; (5) to evaluate experimental vaccines for safety, antigenicity, and effectiveness against naturally occurring respiratory tract disease.

Methods Employed:

Infants and children with respiratory disease and suitable control subjects without such disease are studied at the Children's Hospital, District of

Columbia. The main emphasis is on the use of various tissue culture systems and artificial media for the recovery of viruses and mycoplasmas. All specimens from the patients are inoculated directly into tissue cultures and onto mycoplasma media without prior freezing and thawing, if feasible. In addition to specimens for isolation of viruses and mycoplasma, serial nasal washings are taken to study respiratory tract secretory antibody response.

After the various agents have been identified, their contribution to the different respiratory disease syndromes is estimated by comparing the recovery rate from such groups with that observed for healthy children free of respiratory symptoms. In addition, children with severe respiratory disease admitted to the hospital as well as comparable control subjects, are studied serologically for evidence of infection with the known respiratory viruses and mycoplasmas as well as any new agents which might emerge as potentially important pathogens during the course of the study.

Respiratory viruses which are attenuated by exposure to a chemical mutagen and which exhibit no virulence for adult volunteers are administered to children under carefully controlled conditions in the isolation unit of the Children's Hospital Clinical Research Center. As in other studies, parental consent is obtained in each instance. Vaccinees are observed closely for evidence of illness and specimens are taken for the study of virus excretion pattern, serum antibody response and local secretory antibody response.

Major Findings:

Parainfluenza Viruses - These viruses have continued to be recognized as important pediatric respiratory pathogens since we isolated the first strains from man 16 to 19 years ago. Most of the reports of the epidemiology of parainfluenza virus infection have been concerned with relatively small numbers of infected individuals or a rather short span of study. During the past year, to help rectify this situation the cumulative parainfluenza experience from the Children's Hospital Study 1957-1972 was analyzed. A parainfluenza virus was recovered from 8.3% of 16,400 patients with respiratory disease and from 0.7% of 8,800 control subjects. Type 1 (3.2%) and type 3 (3.4%) viruses were recovered most frequently from respiratory patients, while types 2 (1.3%) and type 4 (0.4%) viruses were encountered less often. The major associations of parainfluenza viruses with disease were (a) type 1 with croup (16.5% isolation frequency) (b) type 2 with croup (9.6%) (c) type 3 with croup (6.2%) (d) type 3 with moderately severe upper respiratory disease (3.9%), and (e) type 1 with moderately severe upper respiratory disease (2.9%). When the results of serologic study (CF) were added to the virus isolation data 41% of patients with croup were found to have been infected with a parainfluenza virus; for pediatric patients with pneumonia the frequency of infection was 14.4%.

The most interesting overall aspect of parainfluenza virus epidemiology involved the alteration of years of prevalence with years of relative scarcity of infection. For example, type 2 and type 3 viruses were prevalent during odd numbered years while type 1 virus was prevalent during even numbered years. The reasons for these 2 year cycles of prevalence are not understood at this time.

Significance to Biomedical Research and the Program of the Institute:

Respiratory disease is the most common ailment of man, particularly during infancy and early childhood when many of the most serious expressions of such illness occur. Detailed knowledge of the agents responsible for respiratory tract disease and an understanding of their natural history are necessary before effective prevention can be achieved.

Proposed Course of Project:

It is planned that this study will continue for a period of years since a large segment of respiratory disease in infancy and childhood remains to be explained--approximately 40%. In addition, viruses responsible for respiratory illness vary in different times and in different localities; therefore, surveillance should continue in order to comprehend the larger picture. Efforts to increase our understanding of the pathogenesis of viral and mycoplasmal lower respiratory tract disease of infancy and childhood will continue.

Honors and Awards:

Director Internationl Reference Laboratory for Respiratory Viruses other than Influenza, World Health Organization.

Director International Reference Laboratory for Mycoplasmas, World Health Organization.

Member International Subcommittee on Taxonomy of the Mycoplasmatales, International Committee on Nomenclature of Bacteria of the International Association of Microbiological Societies.

Served on Editorial Boards of American Journal of Epidemiology and Journal of Infectious Diseases.

Inducted into the National Academy of Sciences, April, 1974.

Participated in review meetings of the Ad Hoc Study Group R & D Command U.S. Army Department of Defense, December, 1973 and March, 1974.

Publications:

Kim, H.W., Arrobio, J.O., Brandt, C.D., Wright, P., Hodes, D., Chanock, R.M., and Parrott, R.H.: Safety and antigenicity of temperature sensitive (ts) mutants of respiratory syncytial virus (RSV) in infants and children. Pediatrics 52: 56-63, 1973.

Kim, H.W., Arrobio, J.O., Brandt, C.D., Jeffries, B.C., Pyles, G., Reid, J.L., Chanock, R.M., and Parrott, R.H.: Epidemiology of respiratory syncytial virus infection in Washington, D.C. I. Importance of the virus in different respiratory tract disease syndromes and temporal distribution of infection. Am. J. Epidemiol. 98: 216-225, 1973.

Parrott, R.H., Kim, H.W., Arrobio, J.O., Hodes, D., Murphy, B.R., Brandt, C.D., Camargo, E., and Chanock R.M.: Epidemiology of respiratory syncytial virus infection in Washington, D.C. II. Infection and disease with respect to age, immunologic status, race and sex. Am. J. Epidemiol. 98: 289-300, 1973.

Brandt, C.D., Kim, H.W., Arrobio, J.O., Jeffries, B.C., Wood, S.C., Chanock, R.M., and Parrott, R.H.: Epidemiology and respiratory syncytial virus infection in Washington, D.C. III. Composite analysis of eleven consecutive yearly epidemics. Am. J. Epidemiol. 98: 355-364, 1973.

Chanock, R.M.: Vaccines for non-bacterial disease of the lower respiratory tract. In Kirkpatrick and Reynolds (eds.) Immunologic Reactions of the Lung, NHLI Monograph, New York, N.Y., Marcel Dekker, Inc., In press.

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Serial No. NIAID 69C
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Etiology of Acute Respiratory Diseases in Adults

Previous Serial Number: Same

Principal Investigator: Dr. Albert Z. Kapikian

Other Investigators: Dr. Robert M. Chanock
Mr. Anthony R. Kalica

Cooperating Units: Mr. W. Lee Cline,(Serology Unit, LID, NIAID)
Dr. James MacLowry,(Clinical Center, Clinical Pathology Microbiology Service)
Dr. Wilmer Dockstader, (Bacteriology Unit Environmental Services Branch)

Contract Support:

Contract Project Number	Project Officer (NIAID)	Contractor	Contractor's Project Director	Annual Funding Level
NIH- 73-C- 229-CC	Dr. Kapikian	Microbiological Associates,Inc.	Mr. M. Vincent	$5,080
NO1-AI-32521 Part B	Dr. Chanock	Flow Laboratories, Inc.	Dr. R. Gilden	$210,000

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	3/12			
Professional:	< 1/12			
Other:	3/12			

Project Description

Objectives: (1) To determine the role of known viruses and bacteria as etiologic agents of acute upper respiratory illnesses in adult populations; (2) to find and characterize new agents which may be human respiratory tract pathogens; (3) to investigate the sensitivity of new experimental cell culture systems for the isolation of rhinoviruses and the more recently described human coronaviruses; (4) to find new methods for the detection of fastidious agents associated with respiratory illness.

Methods Employed:

(A) National Institutes of Health (NIH) Volunteer Study: The current study began on September 9, 1965 and continues to the present. (An earlier similar study began in 1962 and was terminated in 1964). Employees of NIH with respiratory illnesses volunteer a nasal-nasopharyngeal washing, a throat swab, and a blood specimen during the acute phase of a respiratory illness. An additional similar washing and a blood specimen are obtained 3-4 weeks later when the volunteer has recovered from the illness. All of the acute washings are routinely inoculated into cell cultures of primary monkey kidney (MK), Hep-2, HDCS WI-38, primary human embryonic kidney, human embryonic intestine MA 177, and in addition, more recently into BS-C-1 cell cultures also. HDCS WI-26 cultures and human aorta (AT-39) cell cultures were also used earlier in the study. In addition, the first 50 convalescent nasal washings and subsequently 1 of 4 such washings obtained through October 28, 1968, were tested in cell cultures also. Guinea pig erythrocytes are employed in hemadsorption tests in monkey kidney cell cultures for detection of myxoviruses whereas rat erythrocytes are employed similarly in BS-C-1 cell cultures for detection of OC43 related coronaviruses; rat red blood cells have been found to hemadsorb to OC43 virus-infected cell cultures, a finding reported in a previous annual report.

The throat swab is tested for beta (β) hemolytic streptococci (in our laboratory), and in addition, beginning in March, 1969 for streptococci, hemophilus influenzae, and other bacteria, by the Clinical Center Clinical Pathology Microbiology Service (Dr. MacLowry); if β-hemolytic streptococci are isolated they are grouped by the Microbiology Service. In addition, some of the nasal washings have also been tested for staphylococci with subsequent phage typing of recovered organisms (Dr. Dockstader).

In an attempt to find new agents, acute illness specimens which were negative for known viruses in the tissue cultures outlined above, are passaged three times in human embryonic tracheal organ cultures. The third passage harvest is clarified, concentrated, negatively stained with phosphotungstic acid and examined by electron microscopy for the presence of coronavirus-like or other particles. In addition, the harvest from the third passage is routinely inoculated into the cell cultures outlined above to determine if a cytopathogenic agent is recoverable. Recently, the additional technique of "immune electron microscopy" has been employed in an attempt to identify new agents from washings which have failed to yield viral pathogens by other methods. A description of these studies is presented under Project 72A, "New Techniques for Recovery of Respiratory Tract Viruses".

Major Findings:

From September 9, 1965 to April 30, 1974, a total of 722 illnesses were studied in the NIH, LID Common Cold Study. A total of only 14 illnesses

were studied from April 1, 1973 to April 30, 1974. From 0 to 35 were studied each month; some volunteers were in the study more than once. At least one viral agent was recovered or identified from 304 (42%) of the 722 patients; only 2 patients yielded more than one viral agent. β-hemolytic streptococci were recovered from 29 study patients (2 were group A, 6 group B, 5 group C, 3 group F, 7 group K, 2 non-groupable, and 4 not grouped). Three volunteers shed both a β-hemolytic streptococcus (not grouped) and a rhinovirus-like agent, and two shed a β-hemolytic streptococcus (one not grouped and the other non-groupable) and an adenovirus; ten shed both a β-hemolytic streptococcus belonging to groups other than A and a viral agent. Two volunteers shed 2 viruses simultaneously - one both parainfluenza type 3 and an adenovirus, and the other both a rhinovirus and parainfluenza virus type 2. 204 (67%) of the 303 virus isolations were classified as rhinoviruses (202 were acid labile and chloroform stable, 2 have not yet been similarly tested but have characteristic rhinovirus-like CPE, and one (816) was detected by IEM and is included as a rhinovirus since it has rhinovirus-like morphology), whereas 18 (6%) were identified as adenoviruses, 9 (3%) as herpes simplex, 11 (4%) as influenza A2, 7 (2%) influenza B, 6 (2%) parainfluenza type 1, 9 (3%) parainfluenza type 2, 4 parainfluenza type 3, 2 parainfluenza type 4, 6 respiratory syncytial, 23 (8%) coronaviruses (15 "229E-like", one each of OC strains OC37, 38, 43, 48, 692, 700 and 1 untyped presumptive coronavirus tissue culture strain [859])and 5 unclassified viruses (740, 799, 820, 839, 1314).

As shown above, rhinoviruses were by far the most frequently recovered group of agents as they were recovered from 28% of the study patients. However, the epidemiological pattern of rhinovirus infections has been noteworthy as the frequency of such infections has decreased during the first 8 "winter seasons" of the study (December, January and February) in comparison to periods of higher prevalence during preceding "fall seasons" (September, October, and November) (Results from the 1973-1974 fall and winter season are not included since so few patients entered the study). For 8 years combined, rhinoviruses have been recovered from 109 (44%) of the 248 study patients during the "fall periods" and from only 17 (9%) of 199 during the "winter periods". This difference in rhinovirus isolation rate is statistically significant at the .0001 level. During the "winter periods" other known respiratory virus agents were also recovered infrequently using conventional techniques.

Attempts to recover the etiologic agents of respiratory illnesses occurring during these fallow periods have been a major effort of this laboratory. These efforts have been fruitful with the use of two culture techniques, human embryonic tracheal organ cultures and human embryonic intestine MA 177 cell cultures, and in addition the use of the techniques of both immune electron microscopy and conventional electron microscopy. Details on the use of these techniques have been presented in previous annual reports and additional information on their use is presented in project number 72A. In brief, 12 of the 23 definite or presumed coronaviruses identified in this study were

isolated from washings obtained in December, January or February, 9 from washings obtained in March, and two in April. Although specimens obtained in the "winter" or winter-early spring" periods have been tested most intensively, serologic studies from two such periods support the view that coronavirus infections with OC43 and 229E viruses may be important etiologic agents of acute upper respiratory illnesses in adults. In these serologic (CF) studies of paired sera presented in previous annual reports, coronaviruses were the predominant identifiable infecting organism associated with non-influenzal adult respiratory disease.

Paired sera obtained from patients admitted to the study during the 5 year period from September 1965 through August 1970 were tested for serologic (CF) evidence of infection utilizing numerous respiratory agent antigens. In addition, such sera were also tested by the HI technique for evidence of infection with OC43 virus. One hundred ten (20%) of 541 patients developed significant CF and/or HI antibody rises; 10 of the 110 had simultaneous increases in antibody to two agents belonging to different antigenic groups, and one had such increases to 3 such agents for a total of 122 rises. Of the 122 rises, 16 were to the parainfluenza viruses, 3 to adenovirus, 7 to RS virus, 17 to influenza A virus, 9 to influenza B virus, 31 to coronaviruses OC38 and/or OC43 and/or MHV-A59, 32 to coronavirus 229E, 4 to herpes simplex virus and 3 to M. pneumoniae. It is noteworthy that more than one-half (63 of 110) of the CF antibody increases were to the coronavirus antigens.

Further analysis of the data revealed that specimens from 541 study patients were tested by both virus isolation and serologic techniques for evidence of infection during the five-year period, September 1965 through August 1970; such evidence by either virus isolation or serologic (CF, or HI for OC43 virus) techniques or both was found in 300 (55%) of the 541 study patients. In addition, 2 patients had a group A beta-hemolytic streptococcal infection. It was of interest that 62 (11%) of the 541 patients studied by virus isolation and serologic methods had serologic and/or viral isolation evidence of coronavirus infection - 26 (5%) with 229E and/or "229E-like" viruses alone, 23 (4%) with members of the OC38 and/or OC43 and/or MHV-A59 virus group alone, 2 with other presumptive or definite coronaviruses, 5 (1%) with more than one coronavirus only, 4 (1%) with a coronavirus and an agent of a different virus family, 2 with more than one coronavirus and an agent of a different virus family, and one with a coronavirus and M. pneumoniae. Evidence of infection in a single individual with either 2 or all of the OC38, OC43, MHV-A59 group was tabulated as a single coronavirus infection.

Seroepidemiologic Studies of Coronavirus Infections in a College Population -

This study was reported in detail in last year's annual report. In brief, it was found that 19 (17%) of the 110 students developed significant coronavirus CF antibody rises - 12 to 229E and 7 to OC38 and/or OC43 and/or MHV-A59 viruses. In addition, prevalence data indicated that infection with OC43

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or OC43-related agents was very common whereas 229E virus infection occurred less frequently. It appears from this and previously reported studies that OC43 virus infections in adults occur predominantly as reinfections, a pattern similar to that observed with both RS and parainfluenza 3 virus infections in adults. A definitive conclusion concerning the relative importance of the coronaviruses as etiologic agents of acute respiratory diseases must await the development of suitable sensitive methods for their cultivation.

Significance to Biomedical Research and the Program of the Institute -

These studies are aimed at evaluating the role of known viruses and certain bacteria as etiologic agents of adult respiratory diseases and at finding new agents which cause these illnesses. It was noteworthy that with the use of both virus isolation and serologic techniques 55% of the illnesses studied among NIH, LID Common Cold Study volunteers were found to be associated with a viral agent. In addition, the recovery of coronaviruses during certain "winter" and "winter-early spring" periods when non-influenzal respiratory illnesses were prevalent but virus isolation rates paradoxically low was noteworthy. It appears that with the use of new techniques such as organ cultures, experimental cell cultures, immune electron microscopy and conventional electron microscopy, new agents can be identified during such previously fallow periods. Elucidation of the etiology of most upper respriatory illnesses during repeated respiratory disease seasons must be accomplished before attempts at prevention of such illnesses can be fruitful.

Proposed Course of Project:

These studies which attempt to find new agents associated with adult respiratory illnesses will be continued as efforts to identify such agents by using human embryonic organ cultures, experimental cell cultures, and electron microscopy have been rewarding.

Honors and Awards: Invited member of Committee on Communicable Disease Control of the American Public Health Association (Dr. Kapikian). Collaborators and members are revising "Control of Communicable Diseases in Man" for the 12th edition.

Publications: None

Serial No. NIAID 70A
1. Infectious Diseases
2. Respiratory Viruses
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Laboratory Studies of Myxoviruses, Paramyxoviruses and Respiratory Syncytial Virus

Previous Serial No.: Same

Principal Investigators: Dr. David S. Hodes
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Contract Support:

Contract Number	Project Off. (NIAID)	Contractor	Contractor's Proj. Dir.	Annual Funding Level
NO1 AI 32521 Part B	Dr. Chanock	Flow Labs.	Dr. Gilden	$430,000

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	80/12			80/12
Professional:	44/12			44/12
Other:	36/12			36/12

Project Description:

Objectives: (1) To produce conditional lethal temperature-sensitive (ts) mutants of myxoviruses, paramyxoviruses and RS virus for possible use in live attenuated vaccines;(2) to study the genetic basis for attenuation shown by temperature sensitive mutants; (3) to define the relative importance of the various host defense mechanisms in resistance to myxovirus and paramyxovirus infection and disease.

Methods Employed:

Influenza - Influenza A virus suspensions were initially prepared in bovine kidney (BK) tissue culture. Mutagenesis of influenza A/HK/45/68 (H3N2) was accomplished by growth of this virus in BK roller tube cultures in the presence of medium containing 10-2.5M 5-fluorouracil. High titered stocks of virus for use in volunteer studies and for genetic characterization of temperature sensitive mutants were prepared by growing the bovine kidney passaged virus in

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the allantoic sac of either avian leukosis free eggs (for volunteer studies) or of ordinary embryonated eggs (for strains to be used for laboratory characterization).

Genetic analysis of temperature sensitive mutants was accomplished in the following manner. In the plate complementation-recombination technique allantoic fluid suspensions of different mutant pairs were mixed, and inoculated onto monolayers of BK or rhesus monkey kidney (RMK) cells in petri dishes according to the usual plaque technique for the quantitation of influenza virus. During the year this technique was modified to include pre-incubation of the 2 mutants at 4°C for 18 hours prior to inoculation onto the assay monolayer culture. This type of pre-incubation has been shown by Hirst to increase the frequency of genetic interaction between certain mutant pairs. The cultures were incubated at a temperature restrictive for plaque formation for each of the viruses individually. If plaques did develop, the viruses inoculated were assumed to possess genetically distinct lesions, each virus providing for its partner in the cross the gene product (or products) which was defective in the other and thereby enabling plaque formation to occur. Plaques which developed at restrictive temperature contained wild type virus and thus this method of genetic evaluation assayed the occurrence of recombination (most likely by reassortment of the RNA pieces of the virus).

Major Findings:

A. Respiratory Syncytial Virus:

Research on respiratory syncytial virus (RSV) has proceeded along the same general lines as described in the previous annual report. Basically, the research is divided into four separate categories: (1) further characterization of existing ts mutants of the virus; (2) production of new mutant strains for potential use in a live virus vaccine; (3) studies of the mechanisms of viral pathogenesis and of the mechanisms of host resistance; (4) examination of the basic biologic properties of RSV and of related viruses.

(1) Characterization of existing ts mutants - The previous year's work indicated that the 7 existing stable mutants of RSV could be placed in three separate complementation groups. The first group included ts 1, 3, 4, 5 and 6, mutants all produced from the wild type A2 strain by treatment with the chemical mutagen 5 FU. The second group included a single mutant, ts-7, produced by ethylmethanesulfonate. The third group contained ts-2, a 5-FU-induced mutant that exhibited altered plaque morphology and considerable leakiness. Further studies of these mutants indicated that the ts-7 lesion probably affected an earlier function than those affected in the other mutants. Our current work has further clarified physiology of these mutants by elucidating patterns of virus-induced RNA production in mutant-infected cells grown at both permissive temperature (32°C) and restrictive temperature (39°C).

These experiments proceeded according to 2 protocols. In the initial protocol, attempts were made to examine the possibility that the mutant RNA polymerases were temperature sensitive. HeLa cells were infected with ts mutants of RSV and grown at permissive temperature until cytopathic effects were observed. The cultures were then exposed for 90 minutes to 5 μg/ml of actinomycin D at permissive temperature to shutoff cellular RNA synthesis. ^{3}H-uridine, 45 μC/ml was then added and the cultures were incubated for 2 hours at either permissive or restrictive temperatures. Viral specific RNA was then extracted by the SDS-hot phenol method and analyzed by centrifugation through 5-20% sucrose gradients at 40,000 RPM for 4 hours in a Spinco L2-65B preparative ultracentrifuge using an SW 40 rotor. Experiments were completed using each of the mutants. Under the conditions described all mutants were able to incorporate ^{3}H-uridine into RNA in a normal fashion at both permissive and restrictive temperatures. The RNA species produced resembled those previously demonstrated with wild type RSV, i.e., hetero-disperse, slowly-sedimenting RNA's with the main peaks at 10S and 28S. These results seemed to indicate that the function of RNA polymerase at restrictive temperature was not affected by the genetic lesion (or lesions) of any of the ts mutants.

Further experiments were then planned to examine whether some step prior to RNA synthesis was affected in each of the mutants. In this series of studies, cultures were infected with RSV ts mutants as before, but this time incubation of cultures was carried out at both restrictive and permissive temperature rather than only at permissive temperature as in the previous series. Treatment with actinomycin D and labelling with ^{3}H-uridine were also carried out at both temperatures. In these experiments it was found that ts-2, the leaky mutant, incorporated ^{3}H-uridine into RNA with equal facility after incubation at either permissive or restrictive temperature. By contrast, none of the other mutants showed any ability to synthesize RNA after incubation at restrictive temperature. These results indicated that all the mutants except ts-2 were defective in some function occurring prior to RNA synthesis in the viral growth cycle. This finding raised a somewhat paradoxical point. Each mutant virus with the exception of ts-4 had been shown by previous workers in the laboratory to be able to synthesize some viral specific protein (as detected by indirect immunofluorescence and complement fixation) when grown at restrictive temperature. We were able to confirm these results to our own satisfaction. Thus, protein synthesis was occurring at restrictive temperature in the absence of RNA synthesis. Workers in other laboratories have noted similar findings in the case of temperature sensitive mutants of vesicular stomatitis virus. It is possible that the paradox can be explained by assuming that different enzymes or conditions are required for the transcriptive and replicative aspects of viral RNA synthesis. It might be possible that our mutants at restrictive temperature were incapable of RNA replication but capable of initiating a small amount of RNA transcription resulting in the formation of messenger RNA in sufficient quantities to direct detectable amounts of protein synthesis. This amount of RNA might not be sufficient to be detected by the methods employed. The methods employed might be sensitive enough to detect RNA synthesis only when larger quantities of messenger RNA were made or when both RNA transcription and RNA replication were occurring normally. This hypothesis is not testable at the moment.

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(2) Attempts to introduce additional genetic lesions into the ts-1 mutant - As reported last year, considerable effort was spent in attempts to attenuate ts-1 to a greater degree by inducing a second lesion into the genome with the chemical mutagen EMS. It was our belief at that time that the problems encountered might have been caused by passage of the EMS-exposed virus suspensions in BK cells prior to attempts at plaque isolation. Therefore, in subsequent studies, suspensions of ts-1 were given EMS exposure sufficient to cause a 100 to 1000-fold reduction in virus titer and the suspensions were inoculated directly onto BK cell monolayers for plaque isolation. Using this technique we were able to isolate more than 300 plaques initiated by virus surviving EMS exposure.

To simplify the process of identifying possible double mutants, it was decided to employ new criteria. The"shutoff" temperature of a ts mutant is defined as that temperature at which the agent ceases to function normally. Thus ts-1 was found to form plaques normally at 36°C but to be shutoff at 37°C. Since it has been found with influenza virus ts mutants that mutants with lower shutoff temperatures appear to be more attenuated for man, we decided to select new vaccine candidates by identifying clones among the EMS-treated pool which exhibited a shutoff temperature of 36°C. These clones would be expected to be more attenuated than ts-1 (thus circumventing one of the problems seen with ts-1 in clinical trials). It was planned to expose any promising clones to subsequent stress tests to evaluate genetic stability.

Each of the more than 300 clones isolated from the EMS-treated pool was inoculated onto monolayers of HEp-2 cells and incubated for 6 days at 32°C and 36°C. None of the clones showed a lower shutoff temperature than ts-1. It was thus impossible to conclude whether or not remutagenization had occurred.

In view of this failure, we have opened up several new lines of attack in our attempts to develop a more suitable RSV vaccine. First, we have begun experiments using a new mutagenizing agent, nitrosoguanidine (NTG), to induce a second lesion in ts-1. As of this writing we have picked 43 plaques derived by inoculating NTG-treated ts-1 suspensions directly onto BK monolayers. These clones are currently being passed and new clones generated at a rate of 50-100 per week. Isolated clones will be tested for 36°C shutoff temperature as described above.

A second approach to attenuation shows promise. Passaged clones developed from a cold-adapted strain of RSV have been supplied to us by Dr Louis Potash (Flow Laboratories). These clones were found to be temperature sensitive and the temperature sensitivity appears to be genetically stable when the clones are subjected to the stress of incubation at restrictive temperature. These clones will be subjected to further purification by limit dilution and will subsequently be retested for genetic stability at 37°=39°C. Should these latter tests prove successful, studies of experimental infection in hamsters will be carried out to determine whether these mutants are capable of in vivo replication and of inducing an antibody response and a protective effect.

A third approach that has been initiated has been the study of recently obtained bovine strains of RSV. These strains have recently been provided us by veterinary virologists at the University of Missouri (Dr. B.D. Rosenquest) and at Iowa State University (Dr. M.H. Smith). We have begun to produce pools of these agents in BEK cells and we will examine their growth in hamsters as possible vaccines against challenge with human strains of RSV.

(3) Studies on the pathogenesis of RSV disease - These studies have been hampered by our continued inability to find a suitable animal model which is comparable to RSV bronchiolitis in human infants. We have succeeded in confirming, by reconstruction experiments, that injection of hyperimmune serum in hamsters facilitates the clearing of RSV from infected animals' lungs. This finding, however, is in contrast to the finding in human disease and further rules out the hamster as a model of human disease. We have, as yet, been unable to obtain the species of "cotton-rat" employed in Russian studies. Our recent communications with the veterinary groups mentioned above, however, have raised our hopes considerably. These workers have been able to cause clinical disease in normal calves by administering bovine RSV to newborn animals. When similar doses of bovine RSV are administered to calves deprived of colostrum (and hence of maternal antibody), disease does not occur. This situation may have relevance to what happens in infants and we plan to set up collaborative studies in which this experimental disease process will be studied.

(4) Studies on the basic biologic properties of RSV and related viruses - Two main projects have been the center of attention in this area. Recent work at the Molecular Anatomy Program has resulted in the isolation of the RSV envelope "spikes". Using this purified viral product it is hoped to be able to develop a satisfactory radioimmunoprecipitation (RIP) test for the detection of anti-RSV antibody in the nasal secretions of infants. Using the currently available preparation, 2 seronegative goats were given immunizing doses of RSV spikes along with Freund's adjuvant. Both goats responded with increasing titers of anti-RSV neutralizing antibody. In the case of both animals, the anti-RSV neutralizing antibody (as measured by the plaque reduction test currently being used in the lab) was greater than the anti-RSV complement fixing antibody. This would be expected if the neutralizing antibody had its effect on the external RSV envelope and appeared to indicate that an RIP test could be developed successfully. The goat anti-spike antisera was also employed in indirect immunofluorescent studies using rabbit fluorescein conjugated anti-goat globulin. Satisfactory visualization was obtained. The system was then employed to trace the time course of spike antigen production during single cycle infection of HEp-2 cells with RSV. It was found that spike antigen first appeared at roughly 10 hours post-infection, and become maximal by 18-24 hours. Similar results had previously been found using polyvalent anti-RSV sera, indicating that at least some of the viral specific proteins may be synthesized simultaneously during the viral growth cycle.

A considerable effort has been spent examining the effects of a glucose analogue, 2-deoxyD-glucose (2-dG) on the physiology of RSV and of parainfluenza virus type 3 (para 3) and measles viruses. In the case of several other enveloped viruses,

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this agent has been found to inhibit viral growth and cytopathic effects (CPE) by interfering with virus glycoprotein mtabolism. We were able to establish that 2-dG concentrations of 1 mM or greater in the overlay medium caused depression of the replication of RSV, para 3, and measles viruses and dramatically prevented the production of CPE by these agents. 2-dG exerted its effects in the latter half of the viral growth cycles, and removal of the agent at midcycle indicated that the action of the drug was completely reversible. In more defined studies, 2-dG was added to the tissue culture medium at midcycle and virus yield determined by harvesting at 2 hour intervals thereafter. These studies indicated that 2-dG led to a cessation of viral growth within 2 hours, there being no appreciable lag time for the drug's effect. In other studies, 2-dG was added to the overlay medium initially, and then removed in the log phase of viral replication. In these experiments, viral growth proceeded at an accelerated pace following removal of the drug. This indicated that 2-dG blocked a specific step in virus maturation and that earlier functions occurred normally, allowing for accelerated growth when the block was removed. A further indication that 2-dG allowed at least some viral function to proceed normally was seen in immunofluorescent studies where the production of viral antigens (as indicated by indirect immunofluorescence) was found to be unimpaired in the presence of 2-dG at a concentration as high as 10 mM. Results were similar with each of the 3 viruses.

Electron microscopic (EM) studies were carried out using the RSV ts-2 mutant. This agent was employed because it produces the most abundant viral structures when viewed by EM. In the presence of 2-dG, ts-2 was inhibited to the same extent as wild type RSV. EM studies indicated that when 2-dG was added to the overlay medium, the number of virions visualized by EM was markedly reduced. Moreover, those virions which were released seemed to be denuded of surface spike projections. As these spikes have been associated with glycopeptides in the paramyxovirus group, this result was regarded as evidence that 2-dG interferes with glycopeptide metabolism in RSV as it has been shown with other viruses.

We have also attempted to study 2-dG as a possible anti-viral chemotherapeutic agent. So far, we have been unable to alter the course of RSV or para 3 infection in hamsters or influenza A virus infection in mice. These negative results were obtained despite the fact that inhibitory blood concentrations of 2-dG could be obtained by means of parenteral injection of the drug. It was considered likely that the drug did not reach the actual site of viral infection, viz. the respiratory epithelium. Delivery of 2-dG by aerosol, however, did not result in inhibition of virus growth in the lungs.

B. Influenza Viruses:

(1) Isolation and characterization of temperature sensitive mutants of influenza A (H3N2) - The purpose of further isolation and characterization of ts mutants of influenza A is to (1) derive a series of recombinants which have a single lesion or cluster of lesions on one of the functional portions of the influenza A virus genome with the aim of producing ts viruses with lesions specific for

each of the viral genes;(2) derive separate mutants which have a ts defect in the same segment of the influenza A virus genome but which differ with regard to temperature sensitivity; (3) further evaluate ts viruses in seronegative volunteers to explore more fully the relationship between attenuation, site of genetic lesion and level of temperature sensitivity. At present we have 2 types of ts mutants with H3N2 surface antigen (i) four temperature sensitive influenza A viruses, (i.e., ts-1A, ts-1E, ts-1H, ts-2C) derived by recombination of a ts mutant of H2N2 with wild type H3N2 and (ii) a series of 9 different clones of ts viruses which were isolated and cloned from a directly mutagenized stock of H3N2 virus. In the latter group, 1 had a shutoff of 37°C, 6 of 38°C, and 2 of 39°C. These viruses were subjected to genetic analysis by the plate complementation method. Complementation studies entailed using both types of ts viruses described above. The ts-304, and ts-422 viruses each appear not to share lesions with the other 9 mutants and thus each appears to have a single lesion which differs also from those of the 4 distinct ts recombinants (ts-1A, ts-1E, ts-1H, and ts-2C). ts-463 appears to be similar to ts-1H genetically. Two of the other mutants,ts-454 and ts-464 appear to be identical on the basis of complementation with mutants currently available in the laboratory; also these mutants (454 and 464) appear to contain a single lesion and thus constitute a separate complementation group. With the further segregation of lesions (see below) by recombination or by testing of these 2 mutants with other recombinants currently being prepared their true identity should be resolved more completely. The other 4 H3N2 ts mutants (i.e., 303, 315, 528 and 568) appear to have ts lesions in more than one segment of their genomes.

The growth of six of the directly mutagenized H3N2 mutants in the lungs and nasal turbinates of Syrian hamsters was compared with that of the parental H3N2 wild type. In each case, the growth of the mutants was 100 to 10,000 fold less than that of the parental virus. Moreover, relative to the growth of the parental strain the growth of each mutant virus was more greatly diminished in the lungs than in the nasal turbinates. This observation is consistent with the fact that the temperature of the lungs is approximately 37°C while the turbinates are considerably cooler (32-34°C). It is also consistent with the hypothesis involved in deriving these mutants, i.e., that a live ts virus vaccine would replicate sufficiently in the turbinates to mobilize the host defenses, but would be inhibited in its growth in the lungs and thus not cause respiratory illness.

Also the virus isolated from each of these organs was tested for the presence of revertant virus. All of the mutants were selected for their low reversion rate in tissue culture. The studies in hamsters however, afforded an opportunity to study reversion in vivo. Of the 6 ts mutants studied, 3 were relatively stable while 3 had a high reversion rate. The proportion of the virus yield that was revertant was also studied. On the basis of these criteria 2 of the viruses appear to be good potential candidates for use in a live vaccine (ts 454 and ts 528).

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(2) Development of a possible source of reliable tissue culture for genetic studies - A major problem in these studies, and perhaps in the recombination efforts which will be necessary at the time of appearance of new antigenic variants, is the variability of tissue culture (both rhesus monkey kidney and bovine kidney) in supporting plaque formation by influenza A viruses and their genetic recombinants. To circumvent this problem we have worked with H.E.M. Research Associates, Inc. (Rockville, Maryland) to pretest reconstituted aliquots of frozen bovine embryonic kidney tissue. Although such tissue is somewhat less sensitive to influenza A viruses (when compared to rhesus tissue) the cell cultures do appear to be of sufficient quality for both complementation and recombination studies.

(3) ts Recombinants of influenza A(HON1) - We have selected an influenza A/Ann Arbor/Marton/43 (HON1) virus as the wild type virus for recombination with our H3N2 ts viruses for the following reasons: (a) the HON1 virus differs from the H3N2 virus in both surface antigens thereby permitting exploration of the relationship of the ts lesion to the two surface glycoprotein antigens; (b) an HON1 wild type virus was available which had a history of passage only in embryonated eggs, a substrate which is suitable for growth of virus to be used for volunteer studies; (c) all individuals born after the HON1 virus disappeared from circulation in 1947 should be seronegative for the HO hemagglutinin and therefore could serve as volunteers; and (d) a mating with the influenza A (H3N2) ts-1[E] virus would represent a model for the attenuation of the next expected pandemic influenza A virus (H4N3). Because the influenza A (H3N2) ts-1[E] virus shows promise as a live influenza A vaccine strain, a mating of this virus and the HON1 virus was undertaken in bovine kidney cell cultures. Over 300 clones of virus with the HO surface antigen derived from the above cross were examined and 10 percent were found to be temperature sensitive. Fifteen clones from this mating were analyzed in detail with respect to the following characteristics: (a) temperature sensitivity, (b) neuraminidase type, i.e., N1 or N2, (c) genetic composition with respect to the parent ts-1[E] virus (i.e., back cross) and to the other recombinant clones, (d) behavior in animals.

Thirteen of the clones were examined in detail for efficiency of plaque formation (EOP) at 34°, 37°, 38° and 39°C. Nine of the thirteen clones had a shutoff temperature of 38°C, like the parent ts-1[E] virus, and three had a 39°C shutoff temperature. One had a 37°C shutoff temperature.

Fourteen of the clones were examined for neuraminidase type. Nine of the clones were N2 and 5 N1. There was no correlation between EOP and the neuraminidase type of the virus; for example a virus with a shutoff temperature of 38°C could be either N1 or N2. This suggested that the ts-1[E] lesion (or lesions) was not located on the genetic material that coded for the neuraminidase antigen because the ts defect (or defects) segregated independently of the neuraminidase antigen. Previously we had shown that the ts-1[E] lesion was not on the gene or genes which code for the hemagglutinin. The observation that the ts-1[E] virus contains a ts lesion (or lesions) not related to either of the surface antigens is important because it permits one to produce temperature sensitive ts-1[E] recombinant vaccines with any possible combination of hemagglutinin and neuraminidase antigens as new antigenic variants emerge and assume public health importance.

The influenza A (H3N2)-ts-1[E] virus is genetically stable in that it can be grown to $10^{7.5}TCID_{50}$ without evidence of loss of ts phenotype. It is therefore likely that the genome possesses more than one ts lesion. Such putative lesions could be on the same segment of viral RNA or on different segments of RNA. If the latter were the case it should be possible to isolate clones of HON1 ts virus (derived from H3N2 ts-1[E] virus) which exhibit genetic interaction (i.e., recombination) with each other as was observed with the H3N2-ts-1[A], H3N2-ts-1[E] and H3N2-ts-1[H] recombinant viruses derived from the H2N2-ts-1 parent. Two of the fifteen clones analyzed showed evidence of genetic interaction with each other but not with the ts-1[E] parent or most other recombinant clones. This supports the suggestion that the ts-1[E] virus contains ts lesions on different segments of the influenza A virus genome and that these lesions can be segregated independently during mixed infection. By using these segregated ts lesions as probes we are now able to identify the genetic composition of future ts-1[E] recombinant viruses and select those with the desired double lesion. Equally important in this regard, the majority of the HO ts-1[E] recombinant viruses were double mutants and both HON1 ts-1[E] and HON2-ts-1[E] viruses were isolated which contained the 2 ts-1[E] lesions.

Selected clones of HO ts-1[E] recombinant viruses were tested for growth in hamster pulmonary tissue. Each of the HON1 or HON2 ts-1[E] recombinants replicated less well than wild type virus. Viruses with a 38°C shutoff temperature and both ts-1[E] lesions grow less well than viruses with a single lesion or viruses with a 39°C shutoff temperature. These results suggest that selection of a virus which had a 38°C shutoff temperature and which contained both ts lesions of the parent ts-1[E] virus would yield the most desirable type of ts recombinant clone. However, this can be ascertained for the HO ts-1[E] recombinants with certainty only by testing selected recombinants in man. Unfortunately, this cannot be done with these HO ts-1[E] viruses because the parent HON1 wild type virus was not virulent for man.

(4) Recombinants of influenza A/Udorn/307/72 X Hong Kong/68-ts-1[E] - With the occurrence of an antigenic variation of the H3N2 coat of the 1968 Hong Kong strain of influenza A, we embarked on the investigation of the properties of the ts-1[E] temperature sensitive lesion when transferred by recombination from the A/Hong Kong/68-ts-1[E] recombinant into the new 1972 strain. The new wild type strain used, A/Udorn/307/72 is antigenically indistinguishable from the prototype "London flu", A/England/42/72. Recombinant viruses were obtained by dually infecting primary calf kidney cells with A/HK/68-ts-1[E] and A/Udorn/307/72 and treating the progeny virus with an antiserum monospecific for the 1968 H3 hemagglutinin at a dilution which neutralized the 1968 H3 virus but not the 1972 H3. The non-neutralized virus was then plaqued at 33°C. Of 42 plaques picked, 12 (29%) proved to be temperature sensitive. 190 additional plaques are currently under evaluation for the ts characteristic.

Seven of the Udorn/ts-1[E] recombinant clones following triple plaque purification have been extensively studied so far (designated 13, 16, 18, 22, 24, 28, 37). Of these, 4 were restricted in growth at 39°C or higher (39°C shutoff) while 3 were restricted in growth at 38°C or higher (38°C shutoff) which is the phenotype of the HK/ts-1[E] parent. Of these seven clones the 1968 neuraminidase

coat glycoprotein was found on all but one of the 39°C shutoff clones (clone 13). Complementation studies of these clones yielded results consistant with the results being obtained concurrently in the HON1-ts-1[E] recombinant studies (see above). The HON1-ts-1[E] recombinant data suggests that the H3N2 ts-1[E] virus contains two distinct ts lesions which segregated into the R1 and R8 recombinants. The R1 and R8 recombinants recombine with each other thus demonstrating distinct lesions, but these viruses do not recombine with the HK/ts-1[E] parent or with recombinant R11 which presumably received both lesions. Of the 7 Udorn/ts-1[E] recombinants studied none recombined with HK/ts-1[E] or R11, nor did they recombine with each other. Moreover, the 39°C shutoff recombinants (clones 13, 18, 22, 37) behaved like R1 and recombined with R8 but not R1 or R11; the 38°C shutoff recombinants acted like the parent HK/ts-1[E] and did not recombine with it, R11, R8 or R1.

After inoculation of hamsters intranasally, growth of 38°C shutoff clones in the lungs was reduced approximately 300-fold compared to wild type as was the HK/ts-1[E]. The 39°C shutoff clones showed intermediate levels of replication in the lung. Of interest, growth of the ts viruses was more restricted in the lungs than in the cooler nasal turbinates. Infection of hamsters with either 39°C or 38°C type clones provided protection to challenge with wild type virus that was identical to that following wild type infection. Reversion of HK/ts-1[E] and 38°C shutoff Udorn/ts-1[E] recombinants was observed in hamster lungs (37°C) but to a much lesser degree if at all in hamster nasal turbinates where a less strong selection pressure exists due to lower temperature (32°-34°C). In summary, defined ts genetic lesions were passed from the 1968 Hong Kong virus to the subsequently appearing variant A/Udorn/307/72. The physiologic behavior of new recombinant viruses with regard to attenuation and immunogenicity both in vitro and in animals appeared to have been predictable on the basis of the ts lesion or lesions transferred. This hypothesis is currently being evaluated in human volunteers who are receiving the Udorn ts recombinants.

(5) ts Mutants of influenza B virus - Influenza B virus is a respiratory pathogen which infects all age groups and is associated with excess mortality in some years comparable to that caused by influenza A virus. We have therefore begun to develop a live virus vaccine using the methods employed with influenza A virus. We have mutagenized influenza B/Maryland/1959 virus by growing it in the presence of 5-fluorouracil. The purpose for selecting the B/59 virus for the production of the ts viruses was that it differed from the current B viruses significantly, and, therefore, the ts defect from the B/59 ts virus could readily be transferred to current influenza B viruses by genetic recombination.

10 of 378 clones of the mutagenized B/59 virus were found to be temperature sensitive. These 10 clones then were analyzed for their (1) EOP, (2) genetic stability after growth in eggs, (3) genetic relatedness, (4) growth and reversion in the hamster lungs, and (5) ability to provide protection against wild type influenza B/59 virus.

Seven of the viruses had a 39°C shutoff temperature while the remaining 3 had shutoff temperatures of 37-38°C. Only 3 of these viruses maintained their ts characteristic after growth in eggs and therefore were further analyzed in animals for growth, reversion, and protection .

The growth of 4 viruses, ts-78 (39°C), ts-80 (37°C), ts-100 (38°C) and ts-187 (38°C) was evaluated in the hamster lung and nasal turbinate tissue. All ts viruses grew less well than wild type B/59 virus. ts-78 virus with a 39°C shutoff temperature was the least restricted with a 60-fold reduction in virus growth in the lung while ts-80, 100, and 187 showed a 250 to 500-fold reduction. The reduction of virus growth in the nasal turbinates was less than that in the lungs indicating that the replication of these ts viruses in the warmer temperature of the lower respiratory tract was more restricted than that in the cooler passages of the upper respiratory tract. The virus isolated from the pulmonary and nasal turbinate tissue was analyzed for loss of ts phenotype. Of interest, the virus isolated from 16 animals infected with ts-78 (39°C shutoff temperature) maintained its ts phenotype. The ts-100 virus with a 38°C shutoff temperature yielded revertant viruses in the lung of 7 of 11 animals while only 2 of 9 hamsters had nasal turbinates which yielded viruses which lost their ts phenotype. Clones of these viruses picked at 39°C were analyzed for their ts phenotype to see if they represented "leaky" ts viruses, wild type revertants, or viruses with intermediate ts phenotype. Two populations of viruses were found--those which had an EOP similar to wild type virus and those which were intermediate in temperature sensitivity. However, despite the appearance of these viruses with the ability to form plaques at 39°C on the first day after infection, the virus present on day 4 was similar in temperature sensitivity to the ts-100 virus in the original inoculum. Only 3 of 13 and 1 of 8 of the isolates from the lungs or nasal turbinates of the hamsters infected with the ts-80 and ts-187 viruses respectively, yielded wild type virus suggesting that these two viruses were sufficiently stable genetically in hamster pulmonary tissue to be considered for use as donors of ts lesions to future influenza B viruses.

Previous results with influenza A viruses indicated that mutagenesis with 5-FU often resulted in the production of mutant viruses with ts defects in multiple segments of the influenza A virus genome. Similarly, the genetic analysis of the B/59 ts-78, 80, 100 and 187 viruses indicated that some of these mutants contain lesions in more than one segment. Complementation groups cannot be assigned to these viruses because no single ts mutant virus underwent genetic interaction with all the other ts viruses. However, no two mutants are similar by genetic analysis indicating that they are not sister mutants. As with influenza A viruses, these complex genetic relationships will only be clarifed when each ts lesion can be segregated by genetic recombination into separate clones of recombinant virus.

The ability of the ts viruses to provide protection against homologous wild type challenge is being investigated. Preliminary evidence is encouraging in that the temperature sensitive wild type viruses induce homologous protection but fail to restrict the growth of an influenza A virus indicating that the protection is immunologically specific.

(6) Determination of the potential of the influenza A/HK/ts-1[E] virus to produce long-term effects in the hamster - Recent experience in man has indicated that influenza A ts recombinant viruses were attenuated for man. Of the four recombinant clones evaluated in volunteers, the influenza A/HK(H3N2)-ts-1[E]

virus (38°C "shutoff" temperature) possessed many of the characteristics of an acceptable live vaccine strain. One of these desired properties included a pattern of acute infection in man comparable to, but of lesser magnitude than, that caused by wild type influenza A virus. For these reasons, the ts-1[E] and its recombinant progeny viruses were considered for further study in man. Because recent experimental evidence in vitro has demonstrated that temperature sensitive viruses could be isolated from persistently infected tissue culture systems, it was considered necessary to determine if the ts-1[E] virus possessed the capability to initiate a chronic infection in an animal model, the hamster. Hamsters were inoculated intranasally with either influenza A/HK(H3N2)-ts-1[E] virus, wild type Hong Kong virus, or control tissue culture media. The following parameters were evaluated: (1) serum antibody against surface and internal antigens of the influenza A virus; (2) fluorescent (FA) staining of cells obtained from the lungs and brain for the presence of influenza A antigens; and (3) formalin fixed sections of the lungs and brains which were examined for evidence of pathology.

As expected, all animals were infected with the wild type or ts-1[E] virus and the controls remained uninfected. The wild type induced higher serum titers of anti-hemagglutinin (HA) and anti-RNP (internal antigen) antibody than did the ts-1[E] virus. The antibody to the internal antigen was detectable after 1 month of infection but not thereafter in either group whereas antibody to the surface antigen persisted for more than one year after infection. However, the anti-HA antibody titer in both the wild type and ts-1[E] virus infected animals (sampled at bimonthly intervals) decreased at the same rate and at no time was there evidence of a rise in anti-HA or anti-RNP antibody indicating a reactivation of influenza A antigen production.

Using the indirect FA technique, sections of the lungs and brain were examined for the presence of antigen to influenza A virus. It was felt that if the ts-1[E] or wild type viruses had a tendency to initiate a persistent infection in vivo that this state of chronic infection could be detected with appropriate antisera as it can in SSPE and PML in man. In only the wild type virus infected animal was there fluorescence located in the large bronchi of the lungs during the acute infection. FA positive cells were not detected in the lungs thereafter nor in the brains at anytime (also sampled bimonthly).

Since influenza A virus causes only inapparent infection in hamsters, no acute pathological alterations were expected or found. Also no chronic changes were seen in the lungs or brain.

Thus there is no evidence that the wild type or ts-1[E] influenza A virus causes a persistent infection in hamsters using biological criteria that have been used to document such infection (i.e., SSPE and PML) in man. However, to detect whether there is persistence of the nueleic acid of the influenza A wild type or the ts virus in vivo more sophisticated biochemical probes will have to be developed. Although the present study is only into its second year, the results are encouraging in that the ts-1[E] and wild type viruses have behaved similarly. These results, in the context of a similar acute pattern of infection in man, provide a reasonable framework to continue evaluation of these viruses and their progeny in man.

Significance to Biomedical Research and the Program of the Institute:

During the past year considerable progress was made in elucidating the nature of the genetic lesions present in the temperature sensitive (ts) mutants of influenza A virus produced in this laboratory. We were particularly interested in ordering these viruses in terms of genetic relatedness since one of the purposes of this project was to derive mutants which had a single lesion (or cluster of lesions) on only one of the discrete RNA segments of the viral genome. Further it was our intention to produce single mutants with lesions specific for each of the genes of the influenza A virus. Such a set of stable single lesion mutants should prove invaluable as genetic probes for characterization of the lesion or lesions transferred from an older ts vaccine strain to a recently emerged, epidemic strain possessing new surface glycoprotein antigens. For example when the forthcoming influenza A (H4N3) (or H4N2) virus emerges we will transfer the ts-1[E] lesions into the new virus. At that time it will be necessary to authenticate the successful transfer of the ts-1[E] lesions to the new epidemic virus by crossing the H4N3 (or H4N2) ts recombinant with its ts-1[E] parent (i.e., backcross), the mutants containing the 2 single ts-1[E] lesions and finally the other single ts mutants. Availability of single lesion ts mutants should also allow us to define the relationship between site of genetic change in the genome and attenuation for experimental animals and man.

One of the most significant findings of the years involves recognition that the ts-1[E] parent virus contains 2 distinct genetic lesions which segregate independently. The observation that the ts-1[E] virus lesions are not related to either surface antigen is of some importance because it means that one can produce temperature sensitive ts-1[E] recombinant vaccines with any desired combination of hemagglutinin and neuraminidase antigens as new antigenic variants emerge and assume public health importance.

During the past year considerable progress was made in elucidating the nature of the ts lesions present in the ts mutants of RS virus produced in this laboratory. In addition, efforts were intensified to introduce an additional ts lesion into the ts-1 mutant of RS virus in the hope that greater attenuation and increased genetic stability would result. These efforts are of considerable significance to the Institute's program for developing means for prevention of RS disease.

Proposed Course of Study:

Exploration of the genetic lesions of the ts mutants of influenza A virus will continue. The putative "single" ts mutants will be recombined with a wild type virus bearing different surface antigens and the resulting recombinants will be analyzed for the manner in which the lesion segregates with respect to the surface glycoproteins. Genetic characterization of the recombinants should permit us to confirm the single nature of the parent ts virus or alternately, establish the presence of multiple lesions in the parent mutant.

Characterization of the RS ts mutants will continue. Bovine RS virus strains will be evaluated in hamsters and possibly in man for evidence of attenuation. We plan to continue attempts to introduce an additional ts lesion into the ts-1 mutant of RS virus.

Honors and Awards: None

Publications:

Hodes, D.S., Kim, H.W., Parrott, R.H., Camargo, E., and Chanock, R.M.: Genetic alteration in a temperature sensitive mutant of respiratory syncytial virus after replication in vivo. Proc. Soc. Exp. Biol. Med. 145: 1158-1164, 1974.

Hodes, D.S., Schauf, V., and Chanock, R.M.: Isolation of RNA species from HeLa cells infected with respiratory syncytial virus. Proc. Soc. Exp. Biol. Med. 146: 287-290, 1974.

Wright, P.F., Gharpure, M.A., Hodes, D.S., and Chanock, R.M.: Genetic studies of respiratory syncytial virus temperature sensitive mutants. Arch. Gesamte Virusforsch. 41 238-247, 1973.

Kalica, A.R., Wright, P.F., Hetrick, F.M., and Chanock, R.M.: Electron microscopic studies of respiratory syncytial temperature sensitive mutants. Arch. Gesamte Virusforsch. 41: 248-258, 1973.

Serial No. NIAID 70B
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1973 throuth June 30, 1974

Project Title: Classification and Characterization of Rhinovirus Serotypes of Human Origin

Previous Serial Number: Same

Principal Investigator: Dr. Albert Z. Kapikian

Other Investigator: Dr. Robert M. Chanock

Cooperating Units: 7 Collaborating U. S. Laboratories and a WHO international Reference Center (Common Cold Research Unit, Salisbury, Wilts, England, and Clinical Research Center, Harrow, Middlesex, England)

Contract Support:

Contract Number	Project Officer (NIAID)	Contractor	Contractor's Proj. Dir.	Annual Funding Level
NIH-73-C-229-CC	Dr. A.Z.Kapikian	Microbiol. Associates, Inc.	Mr. M. Vincent	$5,080
NO1 AI 32521 Part B	Dr. R.M.Chanock	Flow Laboratories, Inc.	Dr. R. Gilden	210,000

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	10/12			10/12
Professional:	< 1/12			< 1/12
Other:	10/12			10/12

Project Description:

Objectives: (1) To classify the numerous rhinovirus serotypes in order to develop a suitable nomenclature system; (2) to characterize rhinovirus serotypes antigenically; (3) to elucidate the interrelationships between different rhinovirus serotypes.

Methods Employed and Major Findings:

Rhinovirus Collaborative Program - The first and second phases of this collaborative program which was designed to characterize and enumerate the number of distinct rhinovirus serotypes and which was described in detail in previous annual reports instituted a new numbering system for rhinoviruses

based upon extensive cross-testing of a large number of strains. The first phase of this numbering system enumerated 55 distinct serotypes from 1-55 with one subtype. The second phase added 34 additional distinct serotypes thereby extending the numbering system to include rhinoviruses 56 through 89. A third phase of the program included certain second phase viruses which demonstrated one-way or unresolved relationships, and prototype candidate viruses distinct from previously numbered serotypes. We submitted three viruses, 604, 607, and 1169 described in detail in last years' annual report, to the third phase of the program as prototype candidate viruses distinct from previously numbered serotypes. Information concerning the third phase of the program from the rhinovirus reference laboratory of Dr. V. V. Hamparian at Ohio State University (reference laboratory under contract to NIAID) indicates the following: a total of 25 prototype candidate viruses have been submitted to the third phase of the program; reciprocal neutralization tests have indicated that 22 of the 25 are distinct thus increasing the number of rhinovirus serotypes by at least 22; NIH strain 607 was found to be identical to strain SF1662 submitted previously by Dr. J. M. Gwaltney, Jr.; and NIH strains JM-1, 100301, which are holdovers from the second phase, and 604 and 1169 will be included as prototype strains in the third phase. In addition, Dr. Hamparian received from various laboratories 115 presumptive or definite rhinovirus strains which have not been neutralized by antisera to the numbered 90 serotypes but which were not included in the third phase since these strains had not yet been characterized according to some or all of the criteria established by the collaborating laboratories (i.e., virus submitted must be triply "purified"; RNA content, size, ether or chloroform stability, and acid lability must be established; and homologous antisera from "purified" antigen must be submitted). These untypable viruses have now been tested against phase 3 reagents by Dr. Hamparian's laboratory in order to assist in estimating the number of as yet untyped candidate viruses. Results of these tests from Dr.Hamparian indicate (1) 6 of the 115 did not replicate, and 1 was found to be enterovirus-like thereby reducing the number to 108; (2) 41 of the 108 were neutralized by a screening dilution of one or more of the third phase sera, and 38 of the 41 were neutralized by further dilutions of third phase sera (3 of the 41 were not tested in this way); and (3) 67 of the 108 were not neutralized by phase 3 antisera.

Study of Rhinoviruses Recovered from Epidemiologic Studies - 202 acid labile, chloroform stable viruses were recovered from patients enrolled in the NIH, LID Common Cold Study. These agents were classified as presumptive rhinoviruses on the basis of cell culture characteristics, acid lability, and chloroform stability. With the completion of the second phase of the collaborative program and the availability of small amounts of antisera for all numbered serotypes, attempts were made to type some of these rhinovirus isolates. Such a study would not only provide valuable information on the approximate number of uncharacterized serotypes, but also yield epidemiologic information on the prevalence of different serotypes in this community. Thus far, 97 acid labile, chloroform resistant viruses have been tested in neutralization tests with antisera for the 90 numbered rhinoviruses (i.e., 1-89 plus

the subtype). Seventy-seven were typed as follows: 8 as rhinovirus (R) 2; 4 as R7; 3 each as R14, R29, R37, R57; 2 each as R3, R10, R17, R41, R47, R48, R51, R56, R59, R75, R78, R81; and one each as RIA, RIB, R5, R11, R16, R18, R19, R21, R22, R23, R24, R30, R33, R36, R39, R43, R45, R46, R53, R54, R60, R61, R62, R64, R65, R68, R73, R76, and R80. Seventeen could not be typed with antisera for the 90 numbered types and NIH strains 604, 607, and 1169 and these are described in a following section of this report. Acid labile viruses obtained from studies at Junior Village are described in Project 66A.

Characterization of Three Newly Recognized Rhinovirus Types of Human Origin -

In last years' annual report we described the characterization of three rhinovirus serotypes, 604, 607, and 1169 which were distinct from the 90 previously numbered serotypes in reciprocal neutralization tests and which were submitted to the rhinovirus reference laboratory (Ohio State University - Dr. V. V. Hamparian) as potential phase 3 candidate prototype strains; we noted that one strain - 607 - was found by Dr. Hamparian's laboratory to be identical to a strain submitted previously by Dr. J. M. Gwaltney, Jr., whereas the other two were distinct from other phase 3 viruses.

Continuing Characterization of Possible New Rhinovirus Serotypes -

In last years' annual report we reported that 12 additional strains 715, 734, 743, 775, 779, 786, 812, 874, 926, 930, 1010 and 1288 - recovered from washings from volunteers in the NIH, LID Common Cold Study had not been neutralized by antisera to the 90 numbered rhinoviruses and the 3 newly characterized NIH strains 604, 607 and 1169. All but two (812 and 926) were recovered on initial passage in monolayer cell cultures; strain 812 was not recoverable in monolayer cell cultures on initial passage but was recovered in such cultures following 3 passages in HETOC whereas strain 926 induced a CPE after a blind passage in HEK cell cultures. In addition to strain 812 which was reported as having been "purified" by triple terminal dilution in last years annual report, strains 734, 775, 779, 874 and 926 have now also been "purified" similarly. Ten of these 12 strains were submitted to the reference laboratory for testing against the phase 3 candidate prototype strains and other phase 3 viruses which had "one-way" relationships, in order to assist in estimating the number of yet unclassified candidate viruses. In these tests Dr. Hamparian found that (1) NIH strains 715 and 734 were neutralized by strain SF 1585, submitted by Dr. J. M. Gwaltney, Jr.; (2) NIH strain 812 was neutralized by strain SF1803 also submitted by Dr. J. M.Gwaltney , Jr.; (3) NIH strain 874 was neutralized by strain 1321-66 which was submitted by Dr. E. J. Stott and which is a prime strain of R22; and (4) NIH strains 743, 775, 779, 786, 926 and 930 were not neutralized by phase 3 candidate prototype strain antisera or antisera for other phase 3 viruses which had "one-way" relationships.

We have also recovered 5 additional strains, 811, 927, 939 and 995, from washings from volunteers in the Common Cold Study which have not been neutralized by antisera for the 90 numbered serotypes and NIH strains 604, 607 and

LVD
LI
LM
LBV
LMI
LSD

1169. Two of these, 995 and 811, were recovered on initial passage in monolayer cell cultures whereas 3 - 927, 928 and 939 - were not recoverable on initial passage in monolayer cell cultures but were recovered in such cell cultures following 3 passages in HETOC. Further description of these latter 3 strains is given in project 72A.

Significance to Biomedical Research and the Program of the Institute:

Rhinoviruses are the most important known group of viruses to be associated with acute upper respiratory tract illnesses in adults. However, the rapidly increasing number of serotypes and the absence of an acceptable numbering system made interpretation of epidemiological data difficult and also prevented satisfactory epidemiological investigations of rhinovirus infections. The numbering system will permit such studies to be undertaken satisfactorily and these studies will be important in further evaluating the need for and feasibility of producing rhinovirus vaccines.

Proposed Course of Project:

The laboratory portion of the third phase of the collaborative program has now been completed and at least 22 new serotypes will be proposed for addition to the existing numbering system. A fourth phase of the program will be necessary since in a preliminary study it was found by Dr. Hamparian that 67 of 108 strains submitted by various laboratories as being distinct from phase 1 and 2 serotypes were not neutralized by phase 3 antisera as noted above. However it is not known whether or not these untyped strains are comprised of only a few or many serotypes since homologous sera were not available. A fourth phase of this program would be essential to determine the number of rhinovirus serotypes.

In addition, studies on the different antigenic components of individual rhinovirus serotypes will be carried out using the technique of "immune electron microscopy". For this purpose RIA antiserum has been prepared in guinea pigs utilizing both heat inactivated and uninactivated RIA antigen; the effects on viral aggregation by different immunoglobulin groups, such as IgA, will also be investigated. In addition, the typing of rhinoviruses by immune electron microscopy, a method that appears quite promising will be pursued further.

Serial No. NIAID 70C
1. Infectious Diseases
2. Respiratory Viruses
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on Mycoplasmas

Previous Serial No.: Same

Principal Investigators: Dr. Harry B. Greenberg
Dr. Charles Helms
Dr. Robert M. Chanock

Other Investigators: Dr. Benjamin Prescott (LM)

Cooperating Units: None

Contract Support: None

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	44/12		12/12	56/12
Professional:	20/12		12/12	32/12
Other:	24/12			24/12

Project Description

Objectives: (1) To study the biological, biochemical and biophysical properties of mycoplasmas, with particular emphasis on Mycoplasma pneumoniae, the etiologic agent of cold agglutinin positive pneumonia, and to determine the relationship of these properties to virulence; (2) to employ newly developed highly sensitive assays for measurement of antibody to M. pneumoniae in the study of local immunity and epidemiology of infection; (3) to determine the functional role of secretory antibodies produced in the respiratory tract following infection; (4) to further characterize the glycolipid antigens of M. pneumoniae and to evaluate the occurrence of shared antigens in nature; (5) to study the infectivity, stability and protective capacity of ts mutants of M. pneumoniae (strain PI 1428) in the hamster animal model and in man; (6) to study the application of formaldehyde killed vaccine administered locally to hamsters; (7) to evaluate possible functions of secretory antibody directed against M. pneumoniae with specific emphasis on cytoadsorption as a principal factor in virulence.

Methods Employed:

(1) Mycoplasmas were grown on agar plates or in broth suspensions. M. pneumoniae was also grown in Povitsky bottles under conditions in which the colonies became attached to the glass surface.

(2) The complement mediated killing reaction first described by Gale and Kenny for M. pneumoniae was made more sensitive by incubating organisms in the presence of serum for 60 minutes at 4°C prior to the addition of fresh guinea pig serum which served as the source of complement. In the modified killing or mycoplasmacidal (MCT) test the decrease in viability of M. pneumoniae produced by various concentrations of antiserum was determined in the presence of a constant amount of fresh guinea pig serum, and the highest dilution of test serum which produced a 90% killing effect was estimated after logit transformation using a computer program.

(3) A radioimmunoprecipitation method which employed small quantities of reagents (0.025 ml) and microtiter equipment was developed. The organisms were grown in 2 liter Povitsky bottles in the presence of H^3 or C^{14}-oleic acid and C^{14} or H^3-palmitic acid. The cell sheet adhering to the glass surface was washed 3-4 times after removal of the medium. Whole organisms or a 2:1 chloroform methanol lipid extract were filtered through a 450 nm millipore filter and diluted to an activity of 500-1000 cpm per 0.025 ml. Radiolabelled antigen was incubated with different serum concentrations for 60 minutes at 37°C and then overnight at 4°C. The antigen-antibody complexes were precipitated by anti-human globulin. After centrifugation of the precipitates at 1000 rpm for 10 minutes, the radioactivity remaining in the supernatant was determined. From this determination the highest serum dilution which gave a 33% binding of antigen was calculated and considered to be the titer.

(4) Syrian hamsters were inoculated intranasally with either temperature sensitive (ts) or wild type M. pneumoniae. Response of the animals to infection was studied by titration of the number of organisms which had grown in the lungs and nasal turbinates, appearance of MI, CF and mycoplasmaidal antibody in the serum, and lung histopathology. Six weeks to 18 months after initial infection, the animals were challenged intranasally with a virulent wild type M. pneumoniae strain and the response to challenge studied as above.

(5) Syrian hamsters were inoculated intranasally and subcutaneously with 0.2 ml of formaldehyde inactivated M. pneumoniae vaccine or various vegetable glycolipids. These animals were subsequently challenged with wild type organisms and their response to wild type challenge assayed by titration of the quantity of the organism which had grown in the lungs, by study of lung histology and by measurement of antibody response.

(6) Various vegetable and bacterial mixed lipids were isolated by combined chloroform-methanol extraction, sephadex G25 chromotography and DEAE cellulose chromatography. Selected vegetable and bacterial glycolipids were separated from the mixed lipids and purified by additional silicic acid chromatography. The cross reactivity of these glycolipids to M. pneumoniae glycolipids was evaluated by CF, MCT, RIP. Further, certain of the glycolipids were reaggregated with A. laidlawii membrane protein subunits into an artificial membrane and then inoculated into rabbits which were studied for their serologic response.

Major Findings:

Mycoplasma pneumoniae

(A) Secretory antibody response - As shown previously the site of localization of M. pneumoniae appears to be superficial, involving only the epithelial layer of the respiratory passages. The superficial nature of M. pneumoniae infection in the lower respiratory tract suggests that local immune mechanisms (secretory IgA and/or cell mediated immunity) in the respiratory tract should be of greater importance than serum antibody in protection from disease. Recently, the development of a highly sensitive radioimmunoprecipitation (RIP) assay made it possible to detect the development of specific IgA antibody for M. pneumoniae in the nasal secretions of a majority of volunteers infected with this organism. Previous attempts to detect antibody in local secretions had failed but this failure may have resulted from an insensitivity of the serologic methods used.

We tested nasal secretions from 17 men immunized with a formalin inactivated vaccine (OSU 1A) given either parenterally or intranasally. None of the 8 men given this vaccine parenterally developed local IgA rises and only 2 of 9 men given the vaccine intranasally developed a rise in IgA antibody for the organism. In contrast, of 16 men given a live attenuated ts mutant (ts H43), approximately 80 percent showed a significant rise in secretory IgA antibody. These observations suggest that immunization with a live attenuated organism is more effective in stimulating local immunity than an inactivated vaccine given either systemically or locally.

(B) ts Mutants - Temperature sensitive mutants of M. pneumoniae were developed which were restricted in growth in vitro at 36°, 37° or 38°C. At these temperatures the wild type organism grew without restriction. Such mutants were sought with the expectation that their temperature sensitive defects would restrict growth in vivo at the temperature of the lower respiratory tract (37°C), whereas replication would not be impaired in the cooler environment of the upper respiratory passages (32°-34°C).

Four genetically stable temperature sensitive mutants of M. pneumoniae were produced by exposing the organism in 100 µg/ml of N-methyl-N'-nitro-N-nitrosoguanidine. These mutants (ts H9, ts H10, ts H28 and ts H43) and a previously described mutant (ts 640) were temperature sensitive with respect to growth of colonies on agar medium. The gradient of temperature sensitivity was ts H28> ts H43> ts 640> ts H9 and ts H10. In addition, the ts H9 and ts H10 mutants exhibited a decreased capacity to adsorb to receptors on guinea pig red cells.

The ts 640, ts H9, and ts H10 mutants produced an extensive pulmonary infection in the hamster without associated pathological change. The mutants proved to be genetically stable in vivo in that revertants were not recovered from infected hamsters. Furthermore, infection with the ts 640, ts H9 or ts H10 mutant induced significant resistance to a subsequent challenge with virulent wild type M. pneumoniae. Long term animal studies employing ts mutants were

undertaken to evaluate safety and the persistance of the protection induced by ts mutants. Animals infected with wild type M. pneumoniae, or mutants ts 640, ts H9 or ts H10 have now been followed for 1-1/2 years. Results of this study show: (1) the ts mutants are cleared from the hamster at approximately the same rate as wild type organisms;(2) ts mutants appear to induce life-long immunity in hamsters; (3) no adverse effects of the mutants were observed on prolonged follow-up.

Since immunization of man would be more conveniently and comfortably accomplished utilizing the oral route rather than the intranasal route, a study was carried out to see whether oral inoculation of hamsters with M. pneumoniae would result in infection. Preliminary results suggest that oral administration of wild type or ts-640 M. pneumoniae does indeed induce an antibody response. Further experiments to evaluate the protective effect and mechanism of oral immunization with M. pneumoniae are underway.

Preliminary experiments were undertaken to evaluate the infectivity of mutants ts H43 and ts H28 for hamsters. Repeated attempts to infect hamsters with these mutants were unsuccessful despite the fact that ts H43, at least, is infectious for man. Attempts to infect hamsters housed at 0°C, which most certainly lowered upper airway temperature below 37°C were unsuccessful indicating that perhaps the mutants have other defects (host restriction mutations) which limit their growth in hamsters.

(C) Protective effect of inactivated M. pneumoniae vaccine applied topically - Because of the finding that secretory antibody may play a critical role in immunity a preliminary examination of the effect of formaldehyde killed vaccine instilled intranasally was made. Hamsters were given 3 separate doses of 0.2 ml vaccine (lot 10 SU-1A) intranasally or subcutaneously over a 2 week period. Wild type infection and broth control groups were also included. Two weeks following the last administration of vaccine the hamsters were challenged with wild type organism and were sacrificed 10 days later. There was a significant decrease in pulmonary lesions in the intranasally vaccinated animals although little if any effect on growth of the organism in the lungs was seen. The protective effect of IN vaccine as regards prevention of pulmonary lesions was less than that produced by prior wild type or ts infection, but considerably greater than that produced by parenteral administration of inactivated vaccine. Of interest was the observation that prior infection induced significant resistance to subsequent infection, whereas topical administration of inactivated vaccine did not induce resistance to infection of the respiratory tract.

(D) Glycolipid antigenic determinants shared by M. pneumoniae, vegetables, and certain bacteria - This study was prompted by the observation that an unexpectedly large proportion of infants and young children possessed antibody to Mycoplasma pneumoniae in their serum. Since early childhood represents a time of life when the organism is not commonly associated with lower respiratory tract disease, the origin of antibody possessed by infants and young children was not immediately apparent. Such antibody could be the result of clinically

inapparent infection which occurred with high frequency. On the other hand, cross-reactive or shared antigenic determinants might be responsible for some of the childhood serum antibody which reacts with M. pneumoniae. Within the past few years it has been established that the major serologic reactivity of M. pneumoniae resides in a series of glycolipids which are located on the outer membrane of the organism.

In the initial screening tests chloroform-methanol (7:3) eluates containing mixed lipids from 53 vegetables were evaluated for their capacity to fix complement with antibodies to M. pneumoniae or its glycolipids. Forty-three of the vegetables were found to have antigenic activity with the M. pneumoniae immune sera used. Each positive vegetable reacted with the rabbit anti-M. pneumoniae glycolipid serum. Eighteen gram positive bacterial or L forms were also studied and 3 fixed complement with the M. pneumoniae antisera. The positive reactors included Staphlococcus aureus, Staphlococcus aureus L form and Streptococcus hemolyticus group A, type 1 L-form.

Two of the vegetables, and staphylococcus aureus which exhibited a high level of antigen activity were examined in greater detail. Purified glycolipids from carrots and parsnips and staph. as well as artificial membrane reaggregates containing these glycolipids exhibited a high degree of complement fixing activity. The serologically reactive constituents of vegetables and certain gram positive bacteria appeared to be predominantly glycolipid in nature since they reacted to high titer with antibodies directed specifically against the glycolipids of M. pneumoniae. Additional evidence that the carrot, parsnip and staphylococcal glycolipids were closely related to M. pneumoniae glycolipids was obtained in a series of blocking experiments.

Because of the ability of vegetable and bacterial glycolipids to stimulate antibodies against M. pneumoniae experiments were carried out in hamsters evaluating the protective effect of immunization with these cross-reactive antigens. Hamsters immunized either intranasally or parenterally with reaggregated glycolipids were not protected from subsequent infection with wild type organisms. This was not unexpected since killed whole cell preparations are not efficient in stimulating protective immunity.

(E) Radioimmunoprecipitation test for M. pneumoniae antibody - The radioimmunoprecipitation test was modified by use of a crude lipid (2:1 chloroform-methanol) antigen rather than a whole cell antigen. M. pneumoniae organisms were grown as before in the presence of C^{14} or H^3 labelled oleic and palmitic acid. The washed organisms were than extracted in 2:1 CM and the lipid antigen used in the RIP. This modification of the RIP has 2 theoretical advantages (1) previous work has shown that anti-lipid antibody is more closely related to resistance than anti-whole cell antibody, (2) with a lipid antigen available the RIP should be adaptable to the Farr technique which will allow it to be used easily with sera from numerous animal sources.

LVD
LI
LM
LBV
LMI
LSD

Significance to Biomedical Research and the Program of the Institute:

The development and characterization of ts mutants of M. pneumoniae appears to be one of the most promising approaches to prevention of disease caused by this organism. Current findings in this area offer some hope that the ts mutants will prove useful in the control of M. pneumoniae illness.

Of particular significance in our studies was the observation that vegetable glycolipids, either in a suspension of auxiliary lipid such as lecithin or as part of an artificial membrane reaggregate, can stimulate antibodies which react with M. pneumoniae glycolipids and which kill the organism. These findings suggest that glycolipids from vegetables and/or bacteria could be responsible for some of the antibody in human serum which reacts with M. pneumoniae or its glycolipids. However, whether this occurs in nature remains to be established.

Proposed Course of Project:

We plan to continue our investigation of the secretory antibody response in man to M. pneumoniae infection. Attempts will be made to elucidate the chemical nature of the cross-reactive vegetable and bacterial glycolipids. Studies with the ts mutants in hamsters and in man will continue and an attempt will be made to evaluate the importance of cytabsorption to virulence. In this regard the potential role of M. pneumoniae IgA antibody in preventing cytabsorption will be studied. Finally, the feasibility of using a live, ts mutant M. pneumoniae vaccine for prevention of M. pneumoniae disease will be extended to trials in man.

Honors and Awards: None

Publications:

Brunner, H., Greenberg, H.B., James, W.D., Horswood, R.L., Couch, R.B., and Chanock, R.M.: Antibody to Mycoplasma pneumoniae in nasal secretions and sputa of experimentally infected human volunteers. Infect. Immun. 8: 612-620, 1973.

Brunner, H., Greenberg, H., James, W.D., Horswood, R.L., and Chanock, R.M.: Decreased virulence and protective effect of genetically stable temperature sensitive mutants of Mycoplasma pneumoniae. Ann. N.Y. Acad. Sci. 225: 436-452, 1973.

Brunner, H., and Chanock, R.M.: A radioimmunoprecipitation test for detection of Mycoplasma pneumoniae antibody. Proc. Soc. Exp. Biol. Med. 143: 97-105, 1973.

Del Giudice, R.A., Purcell, R.H., Carski, T.R., and Chanock, R.M.: Mycoplasma lipophilum Spec. Nov. Internatl J. Syst. Bacteriol., In press.

Freundt, E.A., Taylor-Robinson, D., Chanock, R.M., Black, F.T., and Purcell R.H.: Proposal for Mycoplasma buccale sp.n. and Mycoplasma faucium sp.n. as names for Mycoplasma orale "types" 2 and 3. Int. J. Syst. Bacteriol., In press.

Greenberg, H., Prescott, B., Brunner, H., James, W., and Chanock, R.M.: Sharing of glycolipid antigenic determinants by Mycoplasma pneumoniae. In Proceedings of the Workshop on New Approaches for Inducing Natural Immunity to Pyogenic Organisms, 1974, In press.

Serial No. NIAID 70D
1. Infectious Diseases
2. Hepatitis Virus
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Laboratory and Epidemiologic Studies of Viral Hepatitis Agents

Previous Serial No.: Same

Principal Investigator: Dr. Robert H. Purcell

Other Investigators: Dr. Stephen M. Feinstone
Dr. Yasuo Moritsugu
Dr. Jonathan Gold
Dr. John M. Peterson

Cooperating Units: Dr. Andrew Morrow (Surgery Branch, NHI, NIH)
Dr. Paul Holland, Dr. Harvey Alter and Dr. Paul Schmidt (CC, Blood Bank, NIH)
Dr. John L. Gerin (MAN Laboratory)
Dr. William London (NINDS)
Dr. James Maynard (CDC)
Dr. C.J. Peters (MARU)
Dr. W. Reeves (MARU)
Dr. John Fox (Univ. Wash., Seattle)
Dr. Thomas Chalmers (CC, NIH)
Dr. Lewellys Barker (FDA)
Dr.Leon Rosen (NIAID, Hawaii)
Dr. Albert Kapikian (NIAID)
Dr. June Almeida (Wellcome Research Lab., England)
Dr. Lesseps Reys (Mozambique)
Dr. Marcel Conrad (Walter Reed)
Dr. Paul Kaplan (Stanford)
Dr. William Robinson (Stanford)
Dr. J. Anthony Morris (FDA)

Contract Support:

Contract Number	Project Off. NIAID	Contractor	Contractor's Proj. Dir.	Annual Funding Level
NOI AI 12079	Dr. Purcell	Mel Labs. Inc.	Dr. Verna	$ 200,757
Interagency Agreement	Dr. Byrne	Oak Ridge Nat. Labs.	Drs. J. Liverman N. Anderson	460,432
Interagency Agreement	Dr. Byrne	CDC	Dr. M. Suggs	70,000
NOI AI 12197	Dr. Purcell	Tulane Univ.	Dr. P. Gerone	24,385
NOI AI 32509	Dr. Tyeryar	Scripps Inst.	Dr. Edgington	79,366

Man Years	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	46/12			96/12
Professional:	60/12	6/12	10/12	76/12
Other:	36/12		6/12	42/12

Project Description:

Objectives: (1) To collect clinical material of high potential infectivity for use in laboratory studies; (2) To develop and test in vitro procedures and animal systems for use in laboratory studies of viral hepatitis; (3) To propagate the viruses of hepatitis A and B in culture or in experimental animals; (4) To study the biochemistry, biophysics, immunology and epidemiology of hepatitis-associated antigens; (5) To develop vaccines or other control measures for viral hepatitis.

Methods Employed:

(1) Clinical materials were collected from patients undergoing major heart surgery at NIH and from various animal and human populations through collaborative studies as described below.

(2) Hepatitis B antigen (HB Ag) was purified by density banding in a cesium chloride gradient and by rate centrifugation in sucrose.

Particles believed to be the virions of hepatitis B were isolated, disrupted with detergent and their internal components analyzed biochemically and immunologically.

(3) Samples containing hepatitis A or B viruses were inoculated into various primate species and the animals were monitored for evidence of hepatitis for six months after inoculation.

(4) A wide variety of serologic techniques were used for seroepidemiologic studies.

Major Findings:

(1) Hepatitis A:

A. Serology - Despite significant progress in recent years in the study of hepatitis B, little progress was made in our understanding of hepatitis A because neither the virus nor infections caused by it could be identified. However, in a collaborative study with Dr. Albert Kapikian of this Laboratory and Drs. Marcel Conrad and Dean Gibson of Walter Reed, we detected a virus-like particulate antigen in the stool of patients acutely ill with hepatitis A and demonstrated its temporal and serologic relationship to hepatitis A infection. The 27 nm particle is believed to be the hepatitis A virus. The antigen was detected, using the technique of immune electron microscopy, in stool samples collected only during the acute phase of infection. Using these

particles as antigen, the development of antibody could be demonstrated during convalescence from hepatitis A infection.

B. Epidemiology - Antibody to hepatitis A antigen was demonstrated with paired serum samples obtained from patients involved in 5 separate naturally occurring outbreaks of hepatitis A as well as from volunteers involved in two studies of experimentally acquired infection. The antigen and its antibody were found to be serologically distinct from hepatitis B antigen-antibody systems as well as from the morphologically similar virus-like antigen associated with acute non-bacterial gastroenteritis.

As part of a collaborative study of hepatitis A jointly funded by NIAID, FDA and CDC, marmosets were inoculated with hepatitis A virus. Approximately 50% of such animals developed clinical evidence of hepatitis. Tests of paired sera from these marmosets for development of antibody to hepatitis A antigen revealed an excellent correlation between clinical evidence of hepatitis and sero-conversion; serologic tests were as sensitive as histologic and biochemical tests for detecting infection.

C. Biophysical characterization - The hepatitis A antigen was found to have a buoyant density in cesium chloride of approximately 1.4 and a sedimentation coefficient in sucrose consistent with its size of 27 nm. These findings, plus those obtained by others, using the marmoset model for characterization of the hepatitis A agent, suggests that this virus may be a parvovirus. More definitive classification must await the determination of the type of nucleic acid it contains.

Biophysical characterization of the hepatitis A antigen is being employed in attempts to purify the particle. Such purified particulate antigen will be used for further characterization and development of simpler and more sensitive serologic tests.

D. Transmission to chimpanzees - Seronegative infant chimpanzees, obtained from a breeding colony jointly funded by NIAID, FDA, and CDC were inoculated with a stool filtrate prepared from stools collected during the acute phase of experimentally acquired hepatitis A infection. The stool specimens were supplied by Drs. Marcel Conrad and Dean Gibson of Walter Reed. Both chimpanzees administered the stool filtrate developed hepatitis A as evidenced by symptoms, elevated transaminase levels, detection of hepatitis A antigen in stool samples collected during the acute phase of infection, and development of antibody to hepatitis A antigen during convalescence. In contrast, the two chimpanzees inoculated with control stool filtrates remained normal. Subsequently, these latter two animals developed evidence of hepatitis A infection following casual contact with the former two animals.

E. Reference reagents - Experimentally infected chimpanzees were plasmapheresed prior to infection and during convalescence. Serum obtained from these plasmaphereses will be serologically evaluated and, if suitable, supplied to the Research Resources Branch of NIAID to be used as an interim reference reagent for hepatitis A antigen.

F. Tissue culture - Efforts to isolate hepatitis A virus in tissue and organ culture will be increased, now that a virus pool of known infectivity and serologic tests for detecting viral multiplication are available.

(2) Hepatitis B:

A. Serology - A micro solid-phase radioimmunoassay specific for the core of the Dane particle (thought to be the hepatitis B virion) was developed. Radioimmunoassay exhibited a high degree of sensitivity and specificity. In a collaborative study with Drs. Kaplan, Greenman and Robinson of Stanford and Dr. Gerin of the MAN Laboratory, the micro solid-phase radioimmunoassay for core antigen was instrumental in localizing viral DNA polymerase activity to the core of the Dane particle, thus strengthening the case for the viral nature of the Dane particle.

B. Epidemiology - Longitudinal studies of post-transfusion hepatitis, carried out collaboratively with Drs. Holland and Alter of the Clinical Center Blood Bank, have continued to yield important information. The frequency of post-transfusion hepatitis has fallen from a high of approximately 50% when unscreened commercial blood was being used to 10% or less, following initiation of an all volunteer blood source policy and a program to screen out all hepatitis B antigen positive blood. The residual post-transfusion hepatitis observed following these changes consists of hepatitis B, presumably caused by infectious blood not detected by the most sensitive hepatitis B antigen screening methods currently available (10%) and non-B hepatitis, of undetermined etiology (90%). Specimens obtained from these studies are being used in an attempt to identify the etiology of non-B post-transfusion hepatitis (see below).

Additional studies of hepatitis B infection among primative native tribes of Panama have been carried out in collaboration with former MARU personnel. Differences in hepatitis B antigen carrier rate have been detected among different native tribes despite evidence of comparable rates of infection, as judged by antigen carrier rate plus antibody rate. These findings suggest that genetic differences may influence the carrier rate of hepatitis B antigen.

Mozambique has the highest incidence of hepatic cell carcinoma (hepatoma) of any place in the world, although other regions of Africa and Asia also have an incidence of hepatoma much higher than Europe and the Americas. Hepatitis B antigen has been found more frequently among hepatoma patients than among non-hepatoma patients in regions of high incidence but not in the United States. To determine if this higher incidence of hepatitis B antigen reflected a higher exposure rate, a higher carrier rate or infection with a specific subtype of hepatitis B antigen, the seroepidemiologic study of four indigenous tribes of Mozambique and a white (Portuguese) population living in Mozambique were studied for hepatitis B antigen and antibody. The frequency of hepatitis B infection, as measured by total frequency of hepatitis B antigen and antibody was similar in the four native populations (46 to 52%), slightly higher among hepatoma patients (64%) but lower among whites residing in Mozambique (21%). In contrast, the frequency of hepatitis B antigen was higher among the two native tribes living in areas of high hepatoma endemicity (15%) than among the two tribes living

in areas of lower endemicity (2-3%). The frequency of hepatitis B antigen was highest among the black hepatoma patients (52%) and lowest among the whites (3%). Every antigen that was subtypeable from the native populations, hepatoma patients and controls was subtype ayw, the type most common in East Africa. In contrast, antigen detected among Portuguese whites in Mozambique was subtype adw, the type most common in Europe. Thus, the hepatitis B virus infection rate appeared to be similar among different African tribes but tribes from areas of high hepatoma endemicity were more predisposed to become hepatitis B antigen carriers.

Continuing studies of hepatitis that occurred among troops in Korea in the 1950's, carried out in collaboration with Dr. Morris of FDA, have revealed that over 80% of such hepatitis was caused by the type B virus. Furthermore, all four recognized subtypes of hepatitis B antigen were associated with apparently non-parenterally acquired hepatitis in Japan, Korea and Okanawa. Since these regions lie in the zone of overlap of the four subtypes, the data strongly suggest that the hepatitis among troops was acquired from the local populations and not as a result of parenteral administration of blood or blood products or use of ellicit parenteral drugs, since hepatitis resulting from these modes of spread would have been associated with subtypes of antigen endemic in the United States (adw and ayw).

C. Transmission to non-human primates -

(1) Rhesus monkeys - Biophysical characterization of hepatitis B virus, using rhesus monkeys as indicators of infectivity, have revealed that the virus appears to be less stable to heat than previously believed (inactivated by exposure to 50°C for 1 hour), inactivated by ether, chloroform and the detergent NP-40, acid-labile, inactivated by cesium chloride but not sucrose and inactivated by an organic iodine compound, sodium hypochlorite and formalin but not by a quarternaryammonium compound. Preliminary *in vitro* neutralization experiments suggest that antibody to the surface antigen and not antibody to the core antigen is the neutralizing (and therefore, protective) antibody following hepatitis B infection. Studies to expand and confirm these preliminary findings are in progress.

(2) Transmission to chimpanzees - In collaborative studies with Dr. Barker of FDA and Dr. Maynard of CDC, pools of hepatitis B virus representing each of the four subtypes of hepatitis B antigen have been prepared and infectivity titers of these pools are being determined in seronegative chimpanzees. The pool of virus representing subtype ayw (the MS-2 pool of Krugman) has an infectivity titer of approximately $10^{7.5}$ chimp infectious $dose_{50}$ (CID_{50}). Infectivity titrations have not been completed for the other three pools, but the adw pool is known to have an infectivity titer of 10^6 or greater CID_{50} per millimliter, and the adr and ayr pools produced hepatitis in chimpanzees when tested undiluted. Collaborative studies to determine if infection with one subtype of hepatitis B virus protects against infection with the second subtype are in progress.

D. Tissue culture - A renewed effort is being made to isolate hepatitis B virus in tissue culture. Methods are based upon new knowledge gained from

animal studies about the stability of hepatitis B virus and new techniques including radioimmunoassays for surface and core antigens and assays for the viral DNA polymerase associated with the Dane particle.

E. Vaccine development - Based upon results of studies of hepatitis B virus in chimpanzees and rhesus monkeys and results of biophysical and immunological studies of hepatitis B antigen, an experimental hepatitis B vaccine has been prepared from purified hepatitis B antigen. Following inactivatiion with formalin, this vaccine was administered to seronegative infant chimpanzees. Following evaluation of the safety and immunogenicity of this vaccine, the chimpanzees will be challenged with live hepatitis B virus to determine if it will protect against infection and illness.

(3) Evidence for Other Hepatitis Agents:

Surgical patients who were followed longitudinally and who developed post-transfusion hepatitis were carefully studied for evidence of infection with hepatitis B virus, cytomeglovirus or Epstein-Barr virus. Only half of the patients had evidence of recent cytomeglovirus infection. However, a comparable proportion of patients with type B hepatitis or no hepatitis at all had evidence of cytomegloinfection. The etiologic relationship of this virus to post-transfusion hepatitis is therefore difficult to determine but probably not significant. None of the patients had evidence of recent Epstein-Barr virus infection. Because it has been assumed that much non-B post-transfusion hepatitis is caused by hepatitis A virus, paired sera from non-B post-transfusion hepatitis patients were tested for development of antibody to hepatitis A antigen. None developed such antibody, and approximately half of those tested had evidence of prior infection with hepatitis A virus. These studies provide evidence for more than two types of human hepatitis virus. Attempts to identify such agents are in progress. These include a search for antigens analogous to hepatitis B antigen and hepatitis A antigen but specific for the new agent and attempts to transmit the agent to non-human primates.

Significance to Biomedical Research and the Program of the Institute:

Hepatitis represents one of the major unsolved infectious disease problems. In addition, changing cultural conditions (the rise of the drug culture) has resulted in a rise in the relative importance of the more serious hepatitis B. Finally, the development of sensitive serologic techniques for the demonstration of prior exposure to hepatitis B has revealed that such infection is much more prevalent in the general population than was previously thought. Thus, hepatitis B is emerging as a public health problem of considerable magnitude. The present program is consistent with the Institute's mission, i.e., responsibility for the study of and, where practicable, the development of vaccines or other suitable methods for the control of infectious diseases. The recent rapid increase in our knowledge of hepatitis B has opened the way for improved control methods. Recent progress in hepatitis A research holds promise for similar increased understanding of this important virus. The program of the Hepatitis Virus Section, LID, NIAID is directed primarily toward the realization of these goals.

Proposed Source of Project

We plan to concentrate our studies of hepatitis B in the following areas: (a) studies of hepatitis B in non-human primates with the twin goals of characterizing the agent of hepatitis B and studying clinical aspects of hepatitis B infection, (b) improvement of serologic tests for HB Ag and other hepatitis-associated antigens and their use in seroepidemiologic studies, (c) extension of biophysical studies of HB Ag and anti-HB Ag interactions, (d) attempts to grow the agents of hepatitis in tissue and organ culture, and (e) through the use of seroepidemiologic and animal model studies, to better understand the parameters of acute and passive immunization against hepatitis, (f) characterize the hepatitis A antigen and determine its relationship to hepatitis A virus, (g) develop simpler and more sensitive tests for hepatitis A antigen and antibody, (h) search for the etiology of non-A, non-B hepatitis and determine its significance by seroepidemiological means.

Honors and Awards:

Invited speaker, U.S.-Japan Cooperative Medical Science Program, Third Revised Program for Workshop on Recent Advances in Epidemiology and Pathogenesis of Viral Diseases, NIH, July, 1973.

Invited Speaker, Gustav Stern Symposium Perspectives in Virology IX, New York, N.Y., February 4 & 5, 1974.

Invited speaker, ICN, UCLA Squaw Valley Symposia, Squaw Valley, California March 24-29, 1974.

Invited speaker, 5th World Congress of Gastroenterology, Hepatitis Symposium, Mexico City, Mexico, October 13-19, 1974.

Invited speaker, Hepatitis B Symposium, Melbourne, Australia, May 2 & 3, 1974.

Invited speaker, Sixth Annual Red Cross Scientific Symposium, May 8, 9, 1974, Washington, D.C.

Invited speaker, American Society for Microbiology meeting, May 6-10, 1974, Chicago, Illinois.

Recipient, Meritorious Service Medal, May, 1974.

Appointed, Head, Hepatitis Virus Section, Laboratory of Infecticus Diseases, March, 1974.

Publications:

Reeves, W.C., Peters, C.J. Lander, J.J., Purcell, R.H., and Johnson, K.M.: Differences in the prevalence of hepatitis associated antigen and antibody among Panamanian Indian populations. J. Infect. Dis. 128: 265-270, 1973.

Alter, H.J., Holland, P.V., Purcell, R.H., Gerin, J.L.: The AusRIA Test: Critical evaluation of sensitivity and specificity. J. Blood, 1973.

Purcell, R.H., Holland, P.V., Wong, D.C., and Morris, J.A.: Viral hepatitis and hepatitis-associated antigen in the Far East. J. Infect. Dis., In press, 1974.

Purcell, R.H., Wong, D.C., Alter, H.J., Holland, P.V.: A microtiter solid-phase radioimmunoassay for hepatitis B antigen. Applied Microbiol. 26: 470-484, 1973.

Purcell, R.H., London, W., Alter, H.J. and Edgington, T.S.: Transmission of hepatitis B virus in rhesus monkeys: Biological characterizations. J. Infect. Dis., In press, 1974.

Kaplan, P.M., Greenman, R.L., Gerin, J.L., Purcell, R.H., and Robinson, W.S.: A DNA polymerase associated with human hepatitis B antigen. J. Virol. 12: 995-1005, 1973.

Lewis, T.L., Alter, H.J., Chalmers, T.C., Holland, P.V., Purcell, R.H., Alling, D.W., Young, D., Frenkel, L.D., Lee, S.L., and Lamson, M.E.: A comparison of the frequency of hepatitis B antigen and antibody in hospital and non-hospital personnel. New Engl. J. Med. 289: 647-651, 1973.

Feinstone, S.M., Kapikian, A.Z., and Purcell, R.H.: Hepatitis A: Detection by immune electron microscopy of a virus-like antigen associated with acute illness. Science 182: 1026-1028, 1973.

Gold, J.W., Alter, H.J., Holland, P.V., Gerin, J.L., and Purcell, R.H.: Passive hemagglutination assay for antibody to subtypes of hepatitis B antigen. J. Immunol. 112: 1100-1106, 1974.

Purcell, R.H.: Viral hepatitis--Identifcation of the A virus. In Sixth Annual Red Cross Scientific Symposium, May 8th and 9th, 1974, Washington, D.C.

Purcell, R.H.: Current understanding of hepatitis B virus infection, and its implications for immunoprophylaxis. Perspectives in Virology, IX, New York, April, 1974.

Serial No. NIAID 70F
1. Infectious Diseases
2. Mycoplasma
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Basic Studies on Mycoplasmas

Previous Serial Number: 207

Principal Investigator: Dr. Joseph G. Tully

Other Investigators: None

Cooperating Units: Dr. M.F. Barile, Bureau of Biologics, FDA, Bethesda, Md.
Dr. I. Kahane, Yale University, New Haven, Conn.

Contract Support: None

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	36/12			36/12
Professional:	12/12			12/12
Other:	24/12			24/12

Project Description:

Objectives: The objectives of these studies cover both basic and applied aspects of mycoplasmas and related wall-free procaryotes, including their nutrition and metabolism, neurotoxins and other biological factors involved in virulence, immunological interrelationships among human and animal strains, and their role in human infection and certain other mixed bacterial, viral and protozoan diseases of man and animals.

Methods Employed:

Mycoplasma toxins and cultural filtrates were prepared from various strains grown under standard cultivation techniques. Purification was accomplished with conventional column chromatography techniques and toxin was assayed by both spectrophotometric and biologic means. Mycoplasma membrane preparations were obtained by subjecting concentrated packed cells to osmotic shock (deionized water). Plant lectins used in membrane binding experiments were analyzed for purity and sugar specificity, and then labeled with radioactive iodine. Organ cultures were prepared from hamster trachea with individual tracheal rings being maintained in Eagle's minimum essential medium (with HEPES buffer) in roller tubes.

Major Findings:

A. Considerable progress in the purification and chemical characterization of neurotoxin from Mycoplasma neurolyticum has been achieved. Earlier studies were hampered by the unstable nature of the toxin and the need to perform chemical and biological assays on the toxin within 24 hrs of preparation. Techniques have now been developed which allow retention of the biological activity of toxic cultural filtrates through procedures involving large volume (100 to 300 ml) pressure ultrafiltration (100 nm), dialysis of filtrates against phosphate buffer (at 4 C), and freeze-drying of dialysates. Toxicity tests in mice have shown the most satisfactory lots of toxin have LD_{50} values of 0.33 to 0.75 mg, and dried toxin remains stable for at least 5 months. Approximately 20 lots of toxin have been prepared with a fully virulent, tissue-culture-passed, Type A strain of M. neurolyticum. The availability of a non-toxic strain (PG-28) of this same serotype provides a useful control for toxin studies and cultural filtrates of this strain have been prepared for comparative studies in purification and toxicity tests. Column chromatography of Type A filtrates have allowed the separation of a toxic protein from other medium components. This toxin passes through the column in the void volume, which suggests a protein with a molecular weight of 200,000 to 800,000. Although the toxin was clearly separated from media proteins, we have observed a decrease (approximately 10-fold) in the specific activity of the isolated and lyophilized toxin. The LD_{50} dose of this material is approximately 3 mg/mouse. Additional tests have ruled out toxin inactivation by chromatography solutions or thermal factors present during the purification procedures. Passage of cultural filtrates of the non-toxic PG-28 strain through G-200 Sephadex columns yields a similar protein at the same point where Type A toxin was recovered. However, the PG-28 protein and all other fractions recovered from filtrates of this strain have not been able to induce neurologic disease. Since uninoculated cultural filtrates do not possess this protein, we believe that while both M. neurolyticum strains may show a similar large molecular weight protein, something in the molecular configuration of the protein recovered from toxic mycoplasmas accounts for the specific activity in the central nervous system. Cultural filtrates have been prepared from strains of M. gallisepticum, M. fermentans, and with virulent (PI 1428) and avirulent (FH) strains of M. pneumoniae. In all these studies, variations in media components, incubation times, and passage levels of mycoplasmas have been investigated in hope of selecting the proper conditions for elaboration of substances with neurotoxic activity. Toxicity tests in mice have not revealed any evidence as yet of neurotoxin in these mycoplasmas.

B. The neurotoxicity of various mycoplasma toxins and cultural filtrates for primates have been carried out in collaboration with Dr. M.F. Barile. Initial trials, performed in 2 to 3 kilogram Rhesus monkeys receiving lyophilized toxic filtrates from M. neurolyticum, showed that quantities in the range of 3 gms/kg (intravenously) were lethal, while 200 mg/kg amounts produced no obvious neurological symptoms. However, Type A toxic filtrates in the range of 1.7 to 2.0 gms/kg (3500 mouse LD_{50} doses) induced immediate central nervous system effects, including flaccid paralysis of the limbs, ataxia, and other signs of incoordination. Recovery occurred within 10-15

minutes and no apparent residual neurological effects were noted, although the post-challenge observation period has been less than 2 weeks. It does not seem likely that media components are involved in the response of monkeys to neurotoxic filtrates since large amounts (2 gms/kg) of other Mycoplasma filtrates (M. pneumoniae PI 1428) have been administered without observing similar symptoms. More extensive control challenges with a non-toxic cultural filtrate (PG-28) will be required, not only to confirm the role of neurotoxin in the observed response but to exclude any possible toxic component present in both avirulent and virulent M. neurolyticum strains.

C. The use of various plant lectins with known specificity for certain carbohydrate residues has yielded important information on the structure of a variety of biological membrane systems. In collaboration with Dr. I. Kahane, we have investigated the binding of several plant lectins to both mycoplasma membranes and whole cells with the thought that this information might supply some insight into the localization of carbohydrates in mycoplasmas and their potential role as special attachment sites for adsorption of mycoplasmas to host tissue cells. The binding of wheat germ agglutinin (WGA), ricin (RCA), and concanavalin A (ConA) to the membranes of approximately 12 Mycoplasma and 3 Acholeplasma species was studied. RCA has specificity for D-galactose residues, ConA for both D-glucose and D-mannose residues, and WGA for N-acetyl-D-glucosamine groups. All mycoplasma and acholeplasma membranes bound the three lectins, although ConA and RCA were always bound more than WGA. The binding appeared specific since significant amounts of each bound lectin were displaced by the specific sugar. Glucose has been identified previously as a predominant component in mycoplasma glycolipids so that the significant amounts of ConA bound to various mycoplasma membranes examined here probably relates to the presence of some glycolipids in all these organisms. The binding of lectins to whole mycoplasma cells was similar to that recorded for membranes, suggesting that binding occurred only at the outer surface of the membrane. Proteolysis of membranes (trypsin and pronase) almost always increased the amount of lectins bound. This observation offers some indirect evidence that glycolipid, hexosamines, or other carbohydrate components in native mycoplasma membranes are partially masked by peptides.

D. Tracheal organ cultures have been used by several groups of investigators to study the pathophysiological effects of a limited number of mycoplasmas. We have utilized hamster tracheal organ cultures to study the pathogenesis of several human mycoplasmas and as a possible in vitro model system for the assay of mycoplasma neurotoxin. Initial comparisons were carried out with virulent (PI 1428) and avirulent (FH) strains of M. pneumoniae. Inoculation of tracheal rings, maintained only in tissue culture medium, with 10^1 to 10^6 colony forming units/ml (CFU/ml) of each strain showed that the ability of mycoplasmas to inhibit ciliary action was directly related to the challenge levels. When virulent and avirulent M. pneumoniae strains were compared at the same challenge level (10^3 CFU/ml), it was noted that virulent mycoplasmas produced complete ciliary blockage after 14 days incubation, while the avirulent strain required 21 days for inactivation of ciliary action. Control tracheal rings examined after 30 days' incubation still showed over 90% of the cilia actively beating. We also confirmed observations recorded earlier that the addition of mycoplasma broth to the tissue culture medium (generally

at ratios of 20% broth to 80% tissue culture medium) greatly decreased the time required for inhibition of ciliary activity. Our findings with a neurotoxic strain of M. neurolyticum indicated that various dilutions (1:2 to 1:32) of cell-free toxin would produce a 50% reduction in tracheal ring ciliary activity within 18-21 days. Dilutions of toxin greater than 1:32 did not significantly reduce ciliary action over a 38-day observation period. Whole cell broth cultures of toxic mycoplasmas (10^2 to 10^8 CFU/ml) also produced a 50% reduction in ciliary movement in 18-21 days, suggesting that neurotoxin alone had a direct inhibitory effect on tracheal ring cilia.

Significance to Bio-Medical Research and the Program of the Institute:

Our current efforts are directed to the role of mycoplasma toxins and other membrane or cell-associated factors in these organisms that may account for their attachment and toxicity to host tissue cells. There are increasing reports of neurological disease associated with M. pneumoniae infections in man and studies on the most well known neurotoxic mycoplasma (M. neurolyticum) can provide basic information on biological factors required for the elaboration of toxin and the chemical nature of this toxin. The findings on neurotoxin tests in primates reported here are the first evidence that the toxin of M. neurolyticum might be involved in neurologic disease in hosts other than rodents. Primates also might be the logical host to assess the role of other mycoplasmas of human origin in neurologic disease.

Carbohydrates, including glycoproteins, glycolipids, and polysaccharides appear to represent only a minor component of the total membrane constituents of mycoplasmas. However, there is increasing evidence that several of these substances play a major role in the serological activity of some pathogenic mycoplasmas and there is speculation that carbohydrate-containing compounds are involved in the adsorption of mycoplasmas to host cells. The indirect evidence offered here to show the presence of exposed carbohydrates on the surface of mycoplasma membranes adds another dimension to the structure of the mycoplasma membrane and suggests further investigation on the role of these compounds in the interaction of mycoplasmas and tissue cells.

Proposed Course of Project:

Further studies are being directed to the isolation and purification of M. neurolyticum toxin. The techniques developed to recover, stabilize, and partially purify the neurotoxin will be applied to other mycoplasmas, especially to those of human origin and species that have been associated with neurologic disease. Additional studies are underway on the susceptibility of primates to other mycoplasma filtrates and the neurotoxin in order to establish more accurate information on quantities of toxin required for host response and their long-term effects on the central nervous system. When sufficient quantities of purified toxin become available these will be evaluated in a variety of animal hosts. Finally, additional effort is being directed to development of adequate in vitro assay techniques for mycoplasmal toxins and other virulence factors.

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Honors and Awards:

Chairman and Member of the Board, FAO/WHO Program on Comparative Mycoplasmology. Participated in Board meeting in Jerusalem, Israel, September 10-11, 1973.

Member, International Subcommittee on the Taxonomy of Mycoplasmatales. Participated in Subcommittee meeting in Jerusalem, Israel, September 7-8, 1973.

Member, Taxonomy Committee, American Society for Microbiology.

Member, Civil Service Committee, American Academy of Microbiology.

Chairman, Program Committee, International Congress on Mycoplasmas of Man, Animals, Plants, and Insects, Bordeaux, France, September, 1974.

Publications:

Tully, J.G.; Biological and serological characteristics of the acholeplasmas. Ann. N.Y. Acad. Sciences, 225: 74-93, 1973.

Saglio, P., L'Hospital, M., Lafleche, D., Dupont, G., Bove, J.M., Tully, J.G. and Freundt, E.A.: Spiroplasma citri gen & sp. nov.: a mycoplasma-like organism associated with "stubborn" disease of citrus. Int. J. Syst. Bacteriol., 23: 191, 1973.

Cole, R.M., Tully, J.G., Popkin, T.J., and Bove, J.M.: Morphology, ultrastructure, and bacteriophage infection of the helical mycoplasma-like organism (Spiroplasma citri gen & sp. nov.) cultured from "stubborn" disease of citrus. J. Bacteriol., 115: 367-386, 1973.

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Cole, R.M., Tully, J.G., Popkin, T.J., and Bove, J.M.: Ultrastructure of the agent of citrus "stubborn" disease. Ann. N.Y. Acad. Sciences, 225: 471-493, 1973.

Tully, J.G., Whitcomb, R.F., Bove, J.M., and Saglio, P.: Plant mycoplasmas; serological relation between organisms associated with citrus stubborn and corn stunt diseases. Science, 182: 827-829, 1973.

Whitcomb, R.G., Tully, J.G., Bove, J.M., and Saglio, P.: Spiroplasmas and mycoplasmas; multiplication in insects. Science, 182: 1251-1253, 1973.

Serial No. NIAID 72A
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: New Techniques for Recovery of Respiratory Tract Viruses

Previous Serial Number: Same

Principal Investigator: Dr. Albert Z. Kapikian

Other Investigators: Dr. Robert M. Chanock
Mr. Anthony R. Kalica

Cooperating Units: Mr. W. Lee Cline, (Serology Unit, LID, NIAID)

Contract Support:

Contract Number	Project Off. (NIAID)	Contractor	Contractor's Proj. Dir.	Annual Funding Level
NO1-AI-32521 Part B	Dr. Chanock	Flow Laboratories, Inc.	Dr. R. Gilden	$210,000
NIH-73-C-229-CC	Dr. Kapikian	Microbiological Associates, Inc.	Mr. M. Vincent	5,080

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	15/12			15/12
Professional:	1/12			1/12
Other:	14/12			14/12

Project Description:

Objectives: (1) To recover new viruses from patients with respiratory disease; (2) to characterize such agents and study their biologic and biophysical properties (3) to develop new techniques for the detection of respiratory tract viruses.

Methods Employed:

Specimens obtained from patients in the NIH,LID Common Cold Study have been studied intensively for the presence of viruses by both conventional and new techniques. The specimens are first examined by routine cell culture methods and only

those which fail to yield viruses are studied further in experimental culture systems - such systems include new cell cultures and human embryonic tracheal (HET) organ cultures. The use of new culture systems has been especially fruitful in the recovery of virus strains belonging to the human coronavirus group of viruses. Sixteen of the 32 described coronavirus strains were first recovered by this laboratory employing new culture systems. However, study of the coronaviruses has been hampered by the lack of convenient and suitable methods for their propagation. Ten of the 32 coronavirus strains were originally isolated in HET organ cultures only, while the remaining 22, all of which were similar to a prototype strain designated 229E, were isolated in monolayer tissue cultures. Since the coronaviruses appear to be important etiologic agents of adult upper respiratory tract illnesses, continued intensive efforts have been made to develop new ways to detect these agents. Progress in the detection of coronaviruses is outlined in the following sections; in addition, the recovery of rhinoviruses by the use of new techniques is also presented.

Major Findings:

Detection of Coronaviruses in HET Organ Culture Harvests by Immune and Conventional Electron Microscopy - The propagation of coronavirus strain 692,(which was distinct from coronaviruses 229E and OC43 by immune electron microscopy),in HET organ culture (OC) and its detection by immune electron microscopy utilizing the patient's convalescent serum as the source of specific antibody was described in detail in a previous annual report. The propagation of coronavirus strain 700 in HET OC and its detection by both immune electron microscopy and conventional electron microscopy was described in last year's annual report.

Attempts to detect additional viral agents, with special emphasis on coronaviruses from NIH Common Cold Study patients' washings which did not yield cytopathic agents in conventional and experimental cell cultures were continued but at a low level. Washings were obtained by instilling 0.85% NaCl into each nostril sequentially, with collection of the fluid emerging from the nostril and occasionally from the mouth also. The washing was then diluted approximately 60% with veal infusion broth containing 0.5% bovine serum albumin. Washings were carried through 3 passages in HET OC and the harvests of the third passage examined for the presence of virus particles, utilizing in addition to conventional electron microscopy, the technique of immune electron microscopy, in which the patient's convalescent serum is the source of specific antibody. Immune electron microscopy was employed so that virus particles would appear in the form of aggregates rather than individual units thereby facilitating the visualization of particles in low-titered preparations.

In the immune electron microscopy procedure 0.8 ml of the HET OC harvest was incubated overnight at 4°C with 0.2 ml of a 1:10 dilution of uninactivated convalescent serum from each respective patient. The mixture was centrifuged at 17,000 rpm for 1-1/2 hrs and the supernate was discarded, and the pellet resuspended with a few drops of distilled water, stained with 3% PTA and placed on a formvar-carbon coated grid; the excess fluid was removed with the edge of a filter paper disc and the grid examined under the electron microscope at a magnification of approximately 44,000. An additional preparation in which PBS was used instead of serum was treated similarly.

No definite coronavirus strains were visualized by these methods since the previous annual report. However, these studies were not given a high priority and as a result, the major use of the electron microscope was apportioned to the gastroenteritis and hepatitis projects. With the anticipated addition of another electron microscope, it is planned that such coronavirus studies can be pursued more intensively.

Detection of Coronaviruses in Nasal-Nasopharyngeal Washings by Electron Microscopy

Washings obtained from 9 patients enrolled in the current or previous NIH Common Cold Study and which had yielded 229E-like viruses in cell culture were examined by electron microscopy in an attempt to determine if coronaviruses could be detected without inoculation of specimens into an in vitro system. Eight were examined by both immune electron microscopy and conventional electron microscopy and one by conventional electron microscopy alone. 0.8 ml of a washing was incubated at room temperature for 1 hr. with 0.2 ml of a 1:5 dilution of a homologous convalescent serum and with 0.2 ml PBS. Further preparation for electron microscopy was as outlined above.

In 2 of 8 washings examined by both immune electron microscopy and conventional electron microscopy, definite coronavirus-like particles were visualized by conventional electron microscopy alone and in the one washing examined by conventional electron microscopy alone definite coronavirus-like particles were also seen. In a fourth washing only a single coronavirus-like particle was seen and this by conventional electron microscopy. Examination of nasal-nasalpharyngeal washings directly by electron microscopy may offer promise in the detection of coronaviruses and other agents as well.

Detection of a Probable Myxovirus by Immune and Conventional Electron Microscopy - In last year's annual report we reported the detection of a probable myxovirus, 740, by examination by immune and conventional electron microscopy of HET OC harvests inoculated with a washing from a patient in the Common Cold Study.

Another myxovirus-like agent, 799, was detected by both immune and conventional electron microscopy. Washings from patient 799 in the NIH Common Cold Study were inoculated into various cell cultures with no resulting CPE or hemadsorption. The washing was carried through 3 HET OC passages and the

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harvests of the 3rd passage were inoculated into cell cultures; no CPE or hemadsorption was observed. The third HET OC harvest was examined by both immune and conventional electron microscopy by methods outlined for detection of coronaviruses in HET OC harvests above, and myxovirus-like particles were visualized by both immune and conventional electron microscopy.

Recovery of Presumptive and Definite Coronavirus strains by Cell Culture Techniques - In a previous annual report we described the recovery of 9 "229E-like" viruses, 8 of which were isolated directly in MA 177 cell cultures only. The MA 177 cell culture appears to be the most sensitive cell culture available for recovery of coronaviruses. In last years annual report we described the recovery in MA 177 and for 3 strains in HEK cell cultures also, of 9 additional 229E-like viruses, 878, 884, 886, 1133, 1142, 1152, 1257, 1259, and 1269, and one presumptive coronavirus strain, 859. No additional cell culture strains of coronaviruses have been recovered (through period ending April 30, 1974).

Utilization of HET OC for Recovery of Rhinoviruses - Washings obtained from patients enrolled in the NIH, LID Common Cold Study are routinely inoculated into a variety of cell cultures as described in project 69C. If a cytopathic or hemadsorbing agent is not detected, the washing is routinely carried through 3 passages in HET OC. The passage from the third OC passage is inoculated into a variety of cell cultures and if no CPE or hemadsorption are observed, the HET OC harvest is examined by the techniques of immune electron microscopy and conventional electron microscopy.

In last years annual report we described the recovery of 5 initially negative in cell culture rhinovirus strains, following inoculation of HET OC harvests into cell cultures. Four of these strains 674, 746, 848, and 880 were typed as rhinovirus (R)78, R75, R76 and R74, respectively, whereas the fifth strain 812 was not neutralized by sera to each of the 90 numbered rhinoviruses and NIH strains 604, 607, and 1169, but has been shown by Dr. Hamparian to be neutralized by strain SF1803 submitted by Dr. Gwaltney (See Project 70B)

We have recovered 9 additional initially negative in cell culture rhinovirus strains, following inoculation of HET OC harvests into cell cultures. A description of the 9 strains follows: (1) Strain 999 was recovered from a washing obtained from a 38 year old male in MA-177 and WI-38 cell cultures following 3 HET OC passages. It produced a rhinovirus-like CPE and was acid labile and chloroform stable. Reisolation attempts from an original washing in cell cultures were unsuccessful. This strain was typed as R61.
(2) Strain 927 was recovered from a washing obtained from a 35 year old male in MA-177 and WI-38 cell cultures following 3 HET OC passages. It produced a rhinovirus-like CPE and was acid labile and chloroform stable. Reisolation attempts from the original washing in cell cultures were unsuccessful. This strain was not neutralized by sera to the 90 numbered rhinovirus types and NIH strains 604, 607, and 1169. It has been "purified" in three terminal dilution passages in WI-38 cultures and the "purified" virus has not been neutralized by antisera to the 90 numbered rhinoviruses and NIH strains 604, 607, and 1169.

(3) Strain 928 was recovered from a washing obtained from a 35 year old male in MA-177 and WI-38 cell cultures following 3 HET OC passages. It produced a rhinovirus-like CPE, and was acid labile and chloroform stable. Reisolation attempts from the original washing in cell cultures were unsuccessful. It was not neutralized by sera to the 90 numbered rhinovirus types and NIH strains 604, 607 and 1169. It has been purified in three terminal dilution passages in WI-38 cell cultures and the purified virus has not been neutralized by antisera to the 90 numbered rhinoviruses and NIH strains 604, 607, and 1169.
(4) Strain 939 was recovered from a washing obtained from a 20 year old female in MA-177 and monkey kidney cell cultures following 3 HET OC passages. This agent produced a rhinovirus-like CPE, and was acid labile and chloroform stable. Reisolation attempts from the original washing in cell cultures were unsuccessful. It was not neutralized by sera to the 90 numbered rhinovirus types and NIH strains 604, 607, and 1169. It has been "purified" in three terminal dilution passages in WI-38 cultures and the "purified" virus has not been neutralized by antisera to the 90 numbered rhinoviruses and NIH strains 604, 607, and 1169.
(5) Strain 1058 was recovered from a washing obtained from a 38 year old male in MA-177 and WI-38 cell cultures following 3 HET OC passages. This agent produced a rhinovirus-like CPE, and was acid labile and chloroform stable. Reisolation attempts from the original washing in cell cultures were unsuccessful. It was typed as R80.
(6) Strain 1049 was recovered from a washing from a 27 year old female in MA-177 and WI-38 cell cultures following 3 HET OC passages. It produced a rhinovirus-like CPE, and was acid labile and chloroform stable. Reisolation attempts from the original washing were successful in MA-177 and WI-38 cell cultures.
(7) Strain 1083 was recovered from a washing from a 22 year old female in MA-349, (semi-continuous human embryonic intestine) cell cultures following 3 HET OC passages. It produced a rhinovirus-like CPE, and was acid labile and chloroform stable. Reisolation attempts from the original washing were successful in MA-177 cell cultures.
(8) Strain 1072 was recovered from a washing from an 18 year old female in MA-177 and WI-38 cell cultures following 3 HET OC passages. It produced a characteristic rhinovirus-like CPE.
(9) Strain 1174 was recovered from a washing from a 50 year old female in MA-177, WI-38, HEK, and MK cell cultures following 3 HET OC passages. It produced a characteristic rhinovirus-like CPE.

Detection of a Presumptive Rhinovirus strain by Immune Electron Microscopy - Strain 816 was visualized by immune electron microscopic study of an HET OC harvest. A washing was obtained from a 42 year old female. It was inoculated into a variety of cell cultures; CPE or hemadsorption was not detected. The washing was then carried through 3 passages in HET OC. The harvest of the 3rd OC passage was reinoculated into a variety of cell cultures; CPE or hemadsorption was not detected. The 3rd OC harvest was examined by immune electron microscopy using a 1:20 dilution of the patient's convalescent serum as the source of antibody according to the method outlined above in the section "Detection of Coronaviruses in HET Organ Culture Harvests by Immune and Conventional Electron Microscopy". It was also examined by

conventional electron microscopy. Aggregates of small rhinovirus-like particles heavily coated with antibody were observed in preparations made from the pooled HET OC harvests and convalescent serum mixture. No such aggregates were observed by conventional electron microscopy.

Significance to Biomedical Research and the Program of the Institute:

The search for new agents using the techniques outlined above is of importance in identifying possible etiologic agents of respiratory disease and as an initial step toward the ultimate goals of prevention and therapy. The recovery of new agents such as those described above attests to the value of this endeavor. Further study of the biologic and biophysical nature of these viruses, and the epidemiology and pathogenesis of diseases associated with them can be expected to bring us closer to these goals.

Proposed Course of Project:

The search will continue for viral agents from patients with respiratory disease using the techniques outlined in this report, and any new systems which appear to be profitable. In addition, further attempts will be made to adapt the organ culture strains to cell culture and to devise new methods for their study.

Honors and Awards:

Invited member of the World Health Organization (WHO), Food and Agriculture Organization (FAO) of the United Nation's Working Team on Coronaviruses in the WHO/FAO Program on Comparative Virology (Dr. Kapikian).

Invited member of International Coronavirus Study Group (Dr. Kapikian).

Invited speaker at the International Associaton of Biological Standardization Symposium on "Immunity to Infections of the Respiratory System in Man and Animals" in London, England. Presentation entitled "Clinical, Epidemiologic and Laboratory Studies of Coronaviruses" (Dr. Kapikian).(April 22, 1974).

Serial No. NIAID 72B
1. Infectious Diseases
2. Respiratory Viruses and Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Study of Respiratory Viruses and Mycoplasmas in Human Volunteers

Previous Serial No.: Same

Principal Investigators: Dr. Brian R. Murphy
Dr. Harry B. Greenberg
Dr. Douglas D. Richman
Dr. Charles Helms
Dr. David S. Hodes
Dr. Robert M. Chanock

Cooperating Units: Dr. Louis Potash, Flow Laboratories
Dr. Richard Hornick and Dr. Y. Togo, and Dr.William Woodward, University of Maryland School of Medicine
Dr. Robert H. Parrott and Dr. H.W. Kim, Children's Hosp., D.C.
Dr. Gordon Douglas, University of Rochester

Contract Support:

Contract Number	Project Off. NIAID	Contractor	Contractor's Proj. Dir.	Annual Funding Level
NO1 AI 32521 Part B	Dr. Chanock Mrs. Mattheis	Flow Labs.	Dr. Gilden	$ 430,000
NOI AI 32503	Dr. Chanock	Univ. of Md.	Dr. Hornick	58,000
NIH 71-2091	Dr. Chanock	Research Fdn. Children's Hosp., D.C.	Dr. Parrott	135,792
NIH 72-2503	Dr. Murphy	University of Rochester	Dr. Douglas	152,110
Interagency Agreement	Dr. Chanock	Lorton Reformatory		51,000

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	81/12			81/12
Professional:	45/12			45/12
Other:	36/12			36/12

Project Description:

Objectives: (1) To evaluate temperature sensitive mutants and recombinants of influenza A virus for evidence of attenuation in adult volunteers; (2) To characterize the serum and local respiratory tract anti-neuraminidase antibody response of man to infection with wild type or attenuated influenza A virus; (3) To evaluate the infectivity and clinical response to ts mutants of M. pneumoniae.

Methods Employed:

(1) Influenza virus - The subjects for these studies were volunteers from the District of Columbia Reformatory in Lorton, Virginia and the Maryland House of Correction, Jessup, Maryland. The influenza virus suspensions used were subjected to extensive safety tests and adventitious agents were not detected. Men were selected on the basis of general good health and absence or low level of serum neutralizing antibody titer for influenza A (H3N2). Informed consent was obtained prior to participation in the study. The men were maintained in isolation in a dormitory for 2 weeks. Volunteers were examined daily by two physicians, and cultures, respiratory secretions, and blood samples were taken when appropriate. The men were inoculated intranasally with $10^{3.0}TCID_{50}$ to $10^{7.0}TCID_{50}$ of virus as a coarse droplet aerosol (DeVilbiss atomizer). Nasopharyngeal washings for virus isolation were collected in veal infusion broth daily and inoculated into rhesus monkey kidney roller tube cultures. Cultures were examined for presence of a hemadsorbing virus every 5 days and discarded after 10 days if negative. Virus isolates were identified by the hemadsorption-inhibition test. Nasal antibody levels were determined by the neutralization technique and serum antibody by hemagglutination-inhibition.

(2) M. pneumoniae - Volunteers were selected for the lowest possible level of serum metabolism-inhibition antibody. 10^7 CFU of the ts-H43 mutant were given by the nasopharyngeal route to 16 adult volunteers. The men were housed in an isolated dormitory and were sampled every other day for presence of mycoplasma in their nasopharynx. Nasal wash, sputum and serum were collected periodically for study of immunological response to the M. pneumoniae mutant.

Major Findings:

A . Influenza A/Udorn/(H3N2-1972) ts-1[E] recombinants - Two of the Udorn/ts-1[E] recombinant viruses were selected for initial investigation, clone 13 which has a 39°C shutoff and clone 16 which has a 38°C shutoff. The former was given to 10 volunteers at the University of Rochester by Dr. Gordon Douglas and his associates and to 10 volunteers at Jessup or Lorton Reformatories by LID investigators. 10^7TCID_{50} of an allantoic cavity-grown suspension of virus was administered intranasally. In each group of 10 men, 2 developed a mild febrile flu-like illness with a maximum temperature less than 38°C. In comparison, wild type influenza A (H3N2-1972) virus produced illness in 50% of seronegative men given $10^{4.0}TCID_{50}$. In previous studies with two 39°C shutoff Hong Kong mutants, ts-1[C] and ts-1[H] also appeared to be attenuated compared

to wild type virus but these recombinants caused mild febrile illness which was unacceptable for a vaccine candidate strain. Studies with 16C1, a 38°C shutoff Udorn/ts-1[E] recombinant, have been more encouraging in that all infections have been silent. However, in studies completed to date only half of the 30 seronegative volunteers given $10^{5.5}TCID_{50}$ of the 16C1 recombinant have developed a serum antibody response. Studies employing a larger inoculum of the 16C1 virus are currently underway. At this stage, the data conforms to the working hypothesis that the defined ts-1[E] lesions are associated with a predictable and transferable level of attenuation for a wild type influenza virus bearing a new surface hemagglutinin antigen. Further, the 13 recombinant which contains only one of the ts-1[E] lesions and which has a 39°C shutoff temperature appears to be less attenuated in man than the 16C1 recombinant which contains both putative ts-1[E] lesions and which exhibits a greater degree of temperature sensitivity.

B. Wild type influenza A/Ann Arbor/Marton/43 (HON1) virus - A major difficulty in evaluating ts mutants of influenza A virus in man for their level of attenuation and immunogenicity involves a scarcity of seronegative volunteers. One possible way to circumvent this is by studying an HON1 virus which could serve as the wild type virus and as the recipient of ts lesions from ts viruses bearing antigens of other subtypes (i.e., H3N2-ts). All individuals born after the HONI virus disappeared from circulation in 1947 should be seronegative for the HO hemagglutinin and could therefore serve as volunteers. This would include the college population. We obtained an HONI virus-influenza A/Ann Arbor/Marton/43 which had been passaged a few times only in eggs and was thus suitable for evaluation in man. It was necessary to evaluate the level of virulence of this wild type virus before we could evaluate the effect of the aquisition of a ts lesion on its virulence. We were disappointed to find that this virus possessed a high human infectious $dose_{50}$ (10^5TCID_{50}). When an inoculum of 10^4TCID_{50} was given, only one of 5 volunteers shed virus, none became ill and none developed serum HAI antibody. At a dose of 10^5 $TCID_{50}$, 2 of 5 men developed mild illness, 2 shed virus, and 3 developed serum antibody. However, none of the 3 men who received 10^6TCID_{50} was infected. These observations are in contrast to our previous findings that $10^{4.5}TCID_{50}$ of influenza A/HK/68 wild type (H3N2) virus (passaged twice in human embryonic kidney culture) produced illness in 6 of 7 men tested. It is likely that the HONI virus which had been passaged in eggs was not highly infectious for man and therefore not a reasonable wild type virus for introduction of ts lesions from influenza A virus subtypes.

Present attempts to obtain a suitable HONI virus include passage of the virus isolated from the above volunteers in bovine embryonic kidney culture and a re-evaluation of its virulence for man. In addition, appropriate investigators in the United States and abroad have been asked if they had other suitable influenza A virus (HON1) strains for evaluation in man for virulence.

(C) Influenza B virus - Temperature sensitive mutants of influenza B/1959 virus are available for recombination with current strains of influenza B virus. It is hoped and expected that influenza B virus will behave like influenza A virus in that the acquisition of a ts lesion through recombination

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will result in attenuation of wild type B virus. Influenza B virus recently has undergone extensive antigenic variation in the past 2 years and two definable variants of the B virus have emerged. One variant influenza B/HK/5/73 is distinctly different from the previously prevalent influenza B/Mass/71 virus strain. The other variant is intermediate in its antigenic composition between the B/Mass/71 and B/HK/73 strains. We obtained a B/HK/5/73 strain isolated and cloned in RIF-free eggs (at 10^{-10} dilution) from the WHO Influenza Centre in England.

We prepared this virus for evaluation in man and found that at 10^5 $TCID_{50}$/man only 2 of 4 volunteers shed virus, but illness did not develop. Since this virus suspension titered only $10^6 TCID_{50}$ it was felt that it did not possess the desired degree of virulence. Passage of the virus and cloning in eggs may have been responsible for its loss of virulence. Therefore, recent strains of B/HK/5/73 like viruses isolated in Washington, D.C. at Children's Hospital and passaged only in tissue culture are being prepared for similar evaluation.

(D) Mycoplasma pneumoniae - Last year we described the production and characterization of a series of temperature sensitive (ts) mutants of M. pneumoniae. Mutants were selected which were restricted in growth on agar at 36°C, 37°C, or 38°C. At these temperatures the wild type organism grew without restriction. Missense mutants of the ts class were sought with the expectation that their temperature sensitive defects would restrict growth in vivo at the temperature of the lower respiratory tract (37°C), whereas replication would not be seriously impaired in the cooler environment of the upper respiratory passages (32°-34°C). Five ts mutants of M. pneumoniae with restrictive temperatures between 36° and 38°C were selected for in vivo studies because of their high degree of in vitro genetic stability. It was shown that these mutants were attenuated for the Syrian hamster and prior infection with those mutants able to replicate in the hamster induced prolonged resistance to subsequent challenge with wild type organism. Importantly, the latter mutants were completely stable genetically in vivo as well as in vitro.

The mutant with intermediate temperature sensitivity (ts-640) was evaluated first in man. In the laboratory, ts-640 was found to be partially restricted in growth on agar at 37°C and completely restricted at 38°C. The mutant was studied in 11 volunteers who lacked pre-existing serum metabolism-inhibiting (MI antibody). Each volunteer was infected and 9 underwent a silent infection. However, 2 of the volunteers developed bronchitis which indicated that the mutant retained sufficient residual virulence to disqualify it as a candidate vaccine strain. Nonetheless, the results of the study were encouraging since ts-640 induced a significant antibody response in the volunteers and the mutant was genetically stable.

The next mutant after ts-640 in the temperature sensitivity gradient is ts-H43. This organism was completely restricted in growth on agar at 37°C and was highly attenuated being non-infectious for hamsters and guinea pigs. In contrast, ts mutants less defective than ts-H43, i.e., mutants with a higher temperature shutoff, produced an extensive, prolonged infection in these rodents. Although mutant ts-H43 lacked infectivity for hamsters and guinea pigs we were

prompted to evaluate this organism in man because a mutant which was slightly less temperature sensitive (ts-640) was infectious for man and retained some virulence.

As described previously, of the 5 genetically stable ts mutants of M. pneumoniae investigated extensively in vitro and in vivo the ts-H43 mutant exhibited the next to the most temperature sensitivity. Unlike the wild type organism which formed colonies on agar medium without restriction between 32°C and 38°C the ts-H43 mutant was unable to produce colonies at 37°C or 38°C. The shutoff temperature of ts-H43 was rather sharp in that colony formation was efficient at 36°C.

Each of the 16 men given the mutant by the intranasal route became infected. Thirteen of the volunteers shed M. pneumoniae, while each of the men developed a significant rise in serum antibody as measured by CF, MI and/or MCT. The initial day of shedding varied considerably--from the first to the 21st day after administration of the mutant. The duration of shedding was also variable ranging from one day to 42 days or greater.

None of the 78 isolates recovered from the volunteers produced colonies on agar at 38°C, whereas colony formation was efficient at 32°C. A more stringent test for genetic alteration of the ts lesion(s) was performed with the last isolate recovered from each infected volunteer. The last isolates were tested for efficiency of colony formation at both 37°C and 38°C in water baths which did not vary in temperature more than 0.05°C. None of the last isolates produced colonies at either 37°C or 38°C. These findings suggest that the ts-H43 mutant did not undergo a change in its ts phenotype during growth in man.

Because of the apparent importance of local immune mechanisms in resistance to M. pneumoniae disease we studied the secretory IgA antibody response of volunteers infected with the ts-H43 mutant. Thirteen of the 16 men were able to produce serial sputum samples. Eleven of these men (84%) developed a significant (i.e., 3-fold or greater) increase in M. pneumoniae IgA antibody as measured by radioimmunoprecipitation using ^{3}H-oleic and ^{3}H-palmitic acid labelled organisms. Eight of the responses were 6-fold or greater. In each instance the maximum secretory response occurred at the end of the second week or later. An increase in secretory antibody in nasal secretions was seen less often than in sputum. Eight of the volunteers developed a 3-fold or greater increase in M. pneumoniae IgA antibody in nasal secretions.

The secretory antibody responses to ts-H43 appeared to occur as frequently and to be of the same magnitude as the responses of volunteers who were infected with a partially attenuated wild type suspension of M. pneumoniae. In addition, the secretory antibody responses of the ts H43 infected volunteers were similar in frequency and magnitude to those described for a larger group of men infected with wild type M. pneumoniae.

None of the men infected with the ts-H43 mutant developed any signs or symptoms of illness during the 28 day period of intensive medical surveillance following administration of the inoculum. There were no elevations of temprature nor did any of the men develop any signs of symptoms involving the respiratory tract.

In summary, in the present study each of 16 volunteers underwent a completely silent infection with mutant ts-H43. Infection of the respiratory tract was extensive enough, however, to stimulate a moderate local secretory antibody response as well as a systemic antibody response. These observations suggest that the ts-H43 organism had achieved the desired balance between attenuation (defectiveness) and antigenicity. The development of a respectable local antibody response was particularly encouraging because of the mounting evidence that local immunological factors in the respiratory tract play a major role in protecting the host against M. pneumoniae disease.

Preliminary observations from a challenge of ts-H43 vaccine with partially attenuated wild type (ts+) M. pneumoniae suggested that the mutant had induced resistance to infection. In the challenge study resistance to illness could not be evaluated, however, infection with wild type (ts+) organisms appeared to be suppressed in men previously given the ts-H43 mutant.

Significance to Biomedical Research and the Program of the Institute:

In practical terms the results of the first volunteer studies with M. pneumoniae mutant ts-H43 are sufficiently encouraging to warrant cautious expansion of evaluation of the mutant in larger numbers of volunteers. Furthermore, while several attempts to use temperature sensitive mutants of respiratory viruses for vaccine purposes have yielded encouraging results, the present effort represents the first application of this technique to free living organisms. The promising results presented here may offer a new approach toward the control of other bacterial respiratory pathogens.

At this time our experience with the 1972 Udorn ts-1[E] recombinants in the laboratory and in seronegative volunteers conforms to our working hypothesis that the defined ts-1[E] lesions are associated with a predictable level of attenuation when transferred into a wild type influenza A virus bearing new surface antigens. These observations have significance for the Institute's program of development of new and more effective means for immunoprophylaxis of influenzal disease.

Proposed Course of Study:

We plan to extend our evaluation of the ts-H43 mutant of M. pneumoniae in a larger group of seronegative adult volunteers. An attempt will be made to infect volunteers by feeding the mutant.

Evaluation of the ts-1[E] recombinants of Udorn 1972 (H3N2) will continue in a larger group of seronegative subjects.

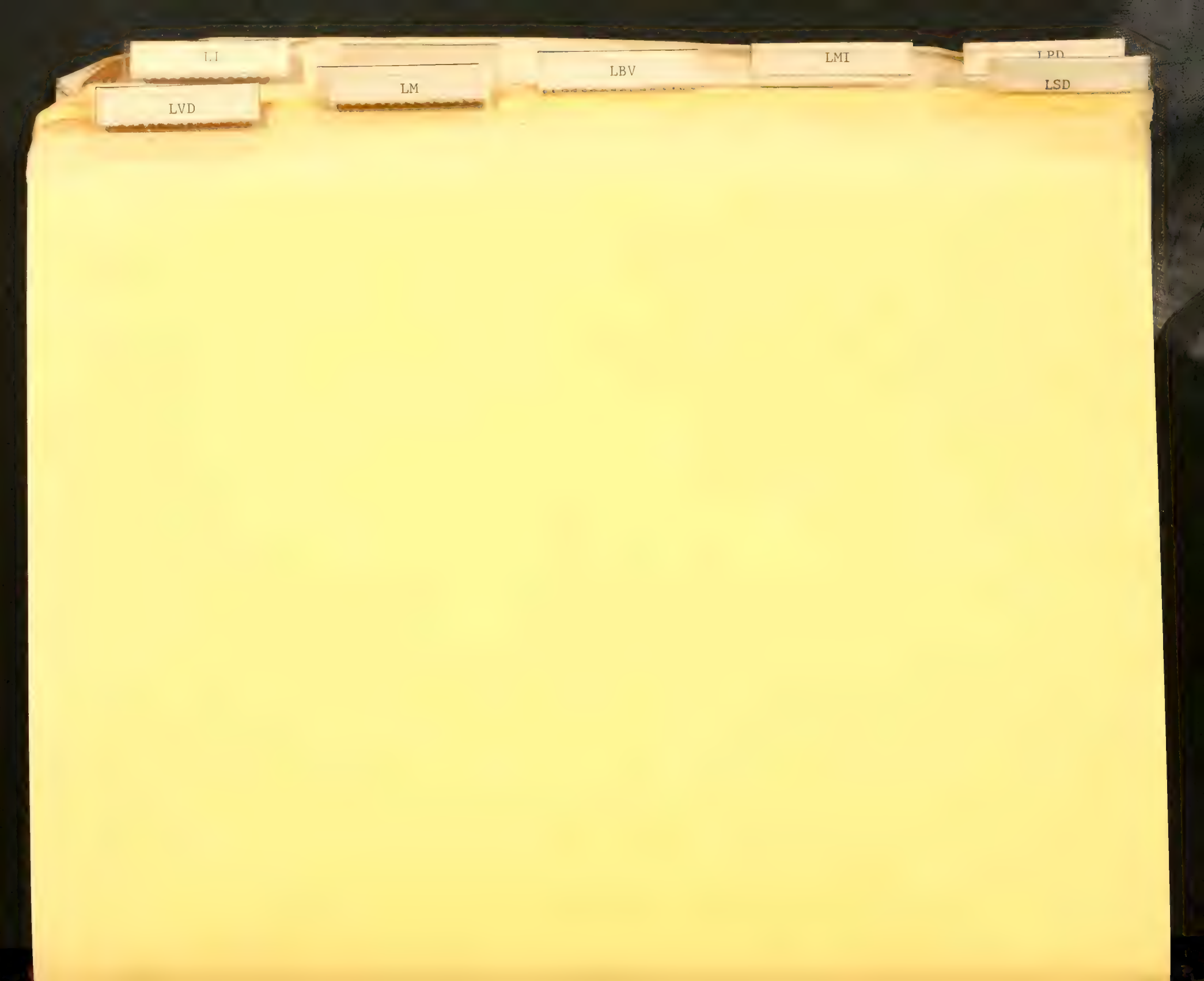
Honors and Awards: None

Publications:

Murphy, B.R., Chalhub, E.G., Nusinoff, S.R., Kasel, J.A., and Chanock, R.M.: Temperature sensitive mutants of influenza virus III. Further characterization of the ts-1[E] influenza A recombinant (H3N2) virus in man. J. Infect. Dis. 128: 479-487, 1973.

Murphy, B.R., Baron, S., Chalhub, E.G., Uhlendorf, C.P., and Chanock, R.M.: Temperature sensitive mutants and influenza virus IV. Production of interferon in the nasopharynx by wild type and a temperature sensitive recombinant virus. J. Infect. Dis. 128: 488-493, 1973.

Murphy, B.R., Hodes, D.S., Nusinoff, S.R., Stewart, S., Tierney, E.L., and Chanock, R.M.: Temperature sensitive mutants of influenza virus. V. Evaluation in man of an additional ts recombinant virus with a 39°C shutoff temperature. J. Infect. Dis., In press.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SUMMARY REPORT

ANNUAL REPORT OF THE LABORATORY OF VIRAL DISEASES, NIAID
July 1, 1973 - June 30, 1974

Dr. Wallace P. Rowe
Chief, Laboratory of Viral Diseases

In general, FY 1974 has been a productive year for LVD, but not without difficulties. The problem of rigidly limited position slots and space has repeatedly caused stresses and friction. These problems are compounded by the very unhealthy atmosphere of insecurity pervading medical research; any immediate threat to "productivity" (interpreted in short-range terms) is recognized as a very real threat to one's career. Fuses are short.

On the brighter side, LVD received two coveted honors, in that Dr. Rowe was awarded the Ricketts Award and the USPHS Distinguished Service Medal.

A major focus of work in LVD is on the genetic transmission of murine leukemia virus in mice. Our previous work has provided compelling evidence that in high-virus strains such as AKR the capacity to produce virus is present in every cell and is inherited as a classical chromosomal element. The crucial question whether the chromosomal loci represented integrated viral genomes or were genes which facilitated expression of RNA copies of the virus had not been settled. By means of biochemical studies we have now been able to resolve this point and open up a large area of inquiry into the nature of these genes.

We have now established that the complete genome of the virus is present in cell DNA, and further, that in virus-positive strains the number of DNA copies of one portion of the viral genome correlates with the rate of spontaneous induction, that the strains which do not yield AKR-type leukemia virus lack this part of the genome, and that inheritance of virus-inducibility is accompanied by inheritance of these DNA sequences. Thus, it is now clear that the virus inducing genes are in fact viral sequences present in the chromosomes. In addition to its implication for virology and carcinogenesis, this finding means that for the first time a eukaryotic gene can be studied simultaneously as a functional and biochemical entity; this could have major applications to the field of molecular genetics of higher organisms.

These studies provide a dramatic example of the impossibility of keeping scientific development within the artificial framework of categorical institutes.

A potentially major development has been made in the program on host defenses, in that a stable and relatively non-toxic interferon inducer has been developed. A complex of poly I:poly C, polylysine, and carboxymethylcellulose, it is resistant to the serum nucleases which hydrolyse poly I:poly C. Since this hydrolysis is probably the major reason for the poor efficacy of poly I:poly C in man, the way may now be open to properly evaluate the clinical use of interferon inducers.

Biochemical detection of murine leukemia virus (MLV) sequences in cell DNA. By using a DNA probe prepared by the reverse transcriptase reaction with AKR-MLV, we have found that mouse cells contain two classes of viral sequences in the DNA. One set is present in all mice, with 7 to 10 copies per haploid genome. The other set is found only in strains which yield AKR-type virus, and the number of copies correlates with the high- or low-virus character of the strain (Chattopadhyay, Lowy, Teich, Rowe).

Lack of allelism of virus-inducing genes in different mouse strains. We have now definitively mapped one of the 3 virus-inducing loci of the high-virus C3H/Fg strain. It is at a completely different site on linkage group I from Akv-1, being adjacent to the Hbb locus. Further, preliminary data on another high-virus strain, C58, indicates that this strain also has a V locus on LG-I, near but not at Akv-1. Since this mouse possesses 3 or 4 V loci, definitive mapping will require several generations of backcrossing to be sure that the LG-I locus has been segregated away from the others. [Rowe, Bremner (Howard University)].

B-tropic MLV inducible from B10.Br strain of mice. A major unsolved problem in the biology of MLV is the origin of B-tropic viruses. Several strains of mice (BALB/c, B10.D2) show high frequency of B-tropic virus in adult life, but embryo tissue cultures yield only N-tropic virus when induced with IUdR. We have now found that one subline of B10.Br rather consistently yields B-tropic virus as well as N-tropic virus from embryo cultures and cell lines therefrom. Each clone has the ability to yield both viruses, and activation of either virus is independent of the other. Capacity to yield B-tropic virus can be transmitted by the male. (Moll, Hartley, Rowe).

Genetics of gs and G_{IX} antigen induction. In cooperation with Sloan-Kettering Institute Scientists, a study of the relationship of the AKR virus-inducing genes to the expression of gs and G_{IX} antigens has been carried out in crosses of C57L (virus negative) and AKR (high virus) mice. The results indicate that gs and G_{IX} antigens can be expressed not only because of infectious virus induced by two AKR V loci, but also by an additional locus which induces these antigens in the absence of infectious virus [Rowe; Ikeda, Stockert, Boyse (Sloan-Kettering)].

MULV with heterologous species host range. A fluorescent antigen induction assay has been developed for detecting and quantitating non-mouse tropic (xenotropic) MULV, a class of viruses first described by LVD scientists in NZB mice and now recognized as being of widespread natural occurrence, probably in all strains of mice. In contrast to the clearly distinct host ranges of mouse-tropic and xenotropic isolates from laboratory mice, viruses of feral mouse origin have been found to replicate in both mouse and heterologous species cell cultures. By limiting dilution purification techniques it has been possible to distinguish a component population capable of growing only in mouse cells. We suspect that these are complex viral populations involving both helper-defective virus interactions and segregating heterozygotes. (Hartley).

lymphocytes transtormed in vitro by Abelson MLV. The Abelson virus, which produces non-thymic lymphomas and plasma cytomas in vivo, has been found to oncogenically transform thymus-independent splenic lymphocytes in vitro, in that infected cultures transplant into mice as lymphomas. This is the first time that a C-type RNA virus has been shown to transform lymphoid cells in vitro. [Sklar, Rowe, Watson (Salk Institute)].

Maternal resistance factor identified. About half of C3H/NeN mothers have been found to induce extremely strong resistance to MLV infection in their offspring. This was demonstrated with both genetically transmitted virus, using C3H♀ X AKR♂ matings, and with exogenous infection (inoculating the babies with virus at birth). Foster-nursing experiments showed that the resistance was transferred via the milk. (Rowe).

IUdR enhancement of adenovirus growth. Pretreatment with IUdR has been found to increase the efficiency of adenovirus infection of partially restrictive cells by 10- to 30-fold. The enhancement is seen with T-antigen induction as well as plaque formation and virus yield, and thus may involve a block to early events such as uncoating or early transcription. (Staal, Rowe).

New virus of house mice. A new agent, apparently viral, has been found in kidney cultures from feral Mus musculus. The agent has many properties of myxoviruses, but does not hemagglutinate or hemadsorb. It has a remarkably broad host range in tissue culture (Manders, Rowe).

Selective inactivation of mycoplasma contaminants of virus suspensions. It has been found that virus preparations contaminated with mycoplasma can be cleaned up by treatment with phenylethyl alcohol, even with delicate enveloped viruses (Staal, Rowe).

Organization of the early SV40 genome. The non-defective Adeno-SV40 hybrids contain only early segments of the SV40 genome. The separated strands of DNA from each of the nondefective hybrids were found to react equally with early + late SV40 DNA. Since the light strand of the hybrid genome contains all the early SV40 DNA sequences while the late SV40 sequences are located on the heavy Ad2 strand, these findings suggest either the presence of equal amounts of early and late SV40 DNA within the early region of the SV40 chromosome, or extensive symmetrical transcription of this region. [Patch, Levine (NCI); Lewis].

Transcription of SV40 DNA segments contained in hybrid virions. SV40 RNA from nondefective hybrid infected cells reacted only with the minus strand of intact SV40 DNA. These RNAs also reacted with the minus strand of the G fragment (a strand containing only antilate SV40 sequences which is produced by digestion of SV40 DNA with H. influenzae restriction enzyme). These findings are comparable to studies on transcription of SV40 DNA integrated into transformed cells in that only the minus strand of the SV40 genome is transcribed in both instances. These findings demonstrate significant alterations in transcription of the SV40 genome after integration into either cellular or viral chromosomes. Such alterations apparently exclude the

transcription of late SV40 sequences [Khoury (LBV); Levine (NCI); Oxman (Harvard); Lewis].

Development of a quantitative assay for Ad2 transformation. Using UV inactivated virions an assay has been devised to study the efficiency of Ad2 in transforming hamster cells. Initial tests demonstrated that the induction of transformed foci is proportional to the dose of the inoculum. Further studies have shown that the efficiency of transforming 5 lots of hamster cells varied from $10^{6.7}$ plaque forming units per focus forming unit (PFU/FFU) to $10^{8.4}$ PFU/FFU. Such an assay will be useful in comparing the efficiency of transformation by Ad2 and the nondefective hybrids as well as among the adeno serotypes. (Lewis).

Transformation of hamster Cells by Ad2 and the nondefective hybrids. Nonhybrid Ad2 and each of the nondefective hybrids transformed hamster kidney cells in tissue culture. All cell lines established from these cultures produced tumors when injected into newborn hamsters. Every cell line tested contained Ad2 T antigens. Only the B55 and ND_4 transformed lines contained SV40 T and TSTA antigens and only the B55 transformed line contained SV40 U antigen. One of the ND_2 transformed lines contains SV40 RNA but no detectable SV40 antigens. These results indicate that interactions between the genomes of nondefective hybrids and the cells they transform are complex and could involve the site of integration, the size of the incorporated viral genome, the transcription or translation of the viral genome, or various combinations of these processes. [Lewis; Levine, Rabson(NCI)].

Evidence for a foamy virus in human tonsils. A culture of human tonsil tissue developed typical foamy virus CPE, and passage of culture fluid to various cell lines produced slight syncytial CPE. The agent could not be maintained in serial passage, however. This provided further evidence that a foamy virus occurs in man (Staal, Rowe).

Stabilization of interferon inducer. A derivative of poly I:poly C has been made that is more resistant than poly I:poly C itself to the hydrolytic action of human serum enzymes. This derivative is a three way complex containing poly I:poly C, polylysine and carboxy methylcellulose. It induces good quantities of interferon in monkeys and chimpanzees, which the parent compound did not. Preliminary studies indicate that its toxicity is no greater than that of poly I:poly C. It is hoped, after suitable toxicological studies to try this compound as an antitumor and antiviral agent in man. (Levy).

Human papovaviruses not a result of polio vaccination. It has been considered possible that the high prevalence of antibody to human papovaviruses, which are related to SV40, resulted from contamination of widely used monkey cell vaccines. Serologic studies on sera collected prior to the use of these vaccines showed a similarly high frequency of antibody (Takemoto, Mullarkey).

Biochemical relation of BK and SV40 viruses. The BK virus, originally isolated from the urine of a renal transplant case, has been biochemically characterized. Six proteins have been identified in the virion and the major protein (VP-1) appears to be smaller than that of SV40. Studies on the DNA of BK virus indicates a relatedness to SV40 to the extent of only

10 to 20%. These studies are being continued to determine which sequences of the DNA of both viruses are shared [Takemoto, Mullarkey, Martin (LBV)].

Isolation of BK virus from patient with immunodeficiency disease and tumor. A virus serologically very similar to BK was isolated from a reticulum cell sarcoma of a Wiscott-Aldrich patient. The virus was also isolated from the patient's urine. Further cases of this disease are being studied virologically. (Takemoto).

New type of virus identified in ameba. A new and unique morphological entity, a beaded structure, has been identified in nuclei of infected E. histolytica. We are currently attempting to isolate this entity and verify our suspicions that it represents a third amebal virus type. [Mattern, Diamond (LPD)].

Induction of interferon by single-stranded viral RNA. Studies of induction of interferon by a single-stranded RNA virus lacking virion-associated polymerase under metabolic conditions where the virus cannot replicate demonstrated full production of interferon. This finding provides evidence that certain single-stranded RNA viruses may produce interferon in the absence of formation of the double-stranded RNA and it indicates areas to examine for new inducers of interferon. (Baron).

Effect of interferon on malaria parasite. A study of the effect of viral interferons on the growth of the avian malarial parasite, P. gallinaceum provides additional supporting evidence for the concept that interferon and interference may affect the growth of malarial organisms in vivo. (Baron).

Evaluation of host defense mechanisms in herpetic keratoconjunctivitis. A study of herpes simplex virus keratoconjunctivitis of the rabbit demonstrated a lack of relationship between local virus titers or local antibody and clinical aspects of the disease. It also indicated little role for either antibody or interferon as natural defenses during this infection of the rabbit. These findings taken together with ongoing studies imply that cellular immunity might be a more important host defense factor for herpes simplex virus infections. (Baron).

Characterization of parvovirus polypeptides. Antisera have now been prepared against purified polypeptides of AAV-1 and the bovine parvovirus, Haden. In comparative serological studies of AAV-1,2 and 3 and Haden virus, cross reactivities and the kinetics of induction of polypeptide antibody response have been defined. Haden virus polypeptide reagents showed no cross reactivity with human parvovirueses. (Hoggan).

AAV carrier cultures. A cell culture line containing AAV-1 genome rescueable by challenge with adenovirus helper has given rise to clones carrying varying amounts of AAV genetic information. Clones producing large amounts of AAV following challenge carry approximately one AAV genome per host cell genome. (Hoggan).

Structural and ultrastructural basis of taste. Continued studies on the architecture and function of mammalian taste buds have shown the presence of a contractile mechanism in the mouse fungiform bud as evidenced by extrusion of fluid from the taste pore upon appropriate stimulus. A potential contractile element has been identified in one of the taste bud cell types. Evidence for acetylcholine being involved in the primary events of taste is supported by morphological and histochemical observations. Synaptic-like vesicles and acetylcholinesterase have been identified by electron microscopy in the taste pore. Preliminary histochemical evidence also indicates that an acetylcholine receptor is located within the pore. These observations have led to an hypothesis suggesting that the taste process might be mediated by an open cholinergic synapse. (Mattern, Paran).

Serial No. NIAID 68H
1. Viral Oncology
2. Viral Diseases
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Immunologic and Other Studies of Animal Leukemia Viruses.

Previous Serial Number: SAME.

Principal Investigator: Dr. M. K. Cook.

Other Investigators: Dr. W. P. Rowe, Miss Johnna Sears.

Cooperating Units: None

Man Years:

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	24/12	0	0	24/12
Professional:	12/12	0	0	12/12
Other:	12/12	0	0	12/12

Project Description:

Objectives: Study of the biology, immunology, and natural history of agents associated with Marek's disease and of Type I (RIF-type) and Type III (Reticuloendotheliosis - CSV) avian leukemia viruses and guinea pig and bovine leukemia viruses.

Methods Employed: Tissue culture systems and standard animal inoculation procedures were employed. Current methods were used to define and characterize the viruses and their antigenic components and the antibody responses to them. Immunofluorescence, complement-fixation, agar-gel precipitation, immuno-electrophoresis, and neutralization tests were employed to define similarities and differences in the antigenic components of avian leukemia viruses, Marek's herpesvirus, and herpes-like and C-type viruses associated with guinea pig and bovine leukemia. Gradient centrifugation, column chromatography, and analysis of viral protein and soluble antigens by polyacrylamide gel electrophoresis were used to separate antigenic components of Marek's herpesvirus.

Major Findings: Attempts were made to define mechanisms of immunologic tolerance, cellular immunity, and lympho-proliferative disease by examining differences in the gs antigens of oncogenic and non-oncogenic Marek's disease

herpesvirus strains. Purification of the gs antigens from a field isolate and a multiple-passaged laboratory strain of Marek's herpesvirus was completed. The antigens were subjected to polyacrylamide gel electrophoresis to attempt to identify the antigenic changes which may be responsible for the immunologic and pathogenic characteristics of field strains after in vitro adaptation. Four peaks were found in the gs antigen of both the field isolate and the laboratory adapted strain. The electrophoretic properties of the proteins in the gs antigenic components of both virus strains shared common features. No qualitative and little quantitative differences in the antigens could be demonstrated by this technique. From these observations one could assume that 1) the changes observed in the immunogenicity of the gs antigens may be due to only minor alterations in the protein structure or 2) that the electrophoretic analysis used was too insensitive to resolve the protein components of the antigens. The changes in the pathogenicity and type of cytopathic effects of the Marek's disease herpesvirus after laboratory passage might be explained by such small changes, but more likely, as with herpes simplex virus, by the differences in the virus strains as to their interactions with infected host cells leading to specification of different membrane proteins and different effects on the interactions of the infected cells.

Proposed Course of the Project: Interaction of C-type viruses and herpesvirus will be studied in chicken and guinea pig systems. High-oncogenic and low or non-oncogenic strains of guinea pig virus will be grown and their gs antigens will be purified and studied. The C-type particle associated with guinea pig leukemia will be characterized. Experiments using herpesvirus alone or multiple infections with herpesvirus and the guinea pig C-type particle will be performed to determine if multiple infection with both viruses is necessary to produce leukemia in guinea pigs and transformation in tissue culture.

Hamster embryo cells treated with UV-irradiated Marek's disease herpesvirus, or UV-irradiated or non-irradiated guinea pig herpesvirus, both in the presence or absence of C-type particles will be studied for transformation and persistence of the viral genomes in the transformed cells which develop. If isolation and high-containment areas are made available, studies with oncogenic and non-oncogenic simian herpesviruses and C-type particles associated with them will be attempted.

Hamster embryo cells seem to be extremely sensitive in undergoing morphological cellular transformation after treatment with herpesviruses. At least seven species (man, mice, rats, guinea pigs, cattle, monkeys, and chickens) and the hamster itself harbor C-type particles. We will attempt to study the pecularities of the hamster tissue culture cells and the role, if any, of the C-particles present in hamster transformed cells.

Significance to Biomedical Research and the Program of the Institute: The guinea pig herpesvirus seems to be a much more useful laboratory tool then the Marek's disease herpesvirus of chickens. The infectivity titers of the guinea pig virus are 100-1000X those obtained with the chicken herpesvirus, and the guinea pig virus is considerably more stable. The purification of

the gs antigens from oncogenic and non-oncogenic strains should be easier, and production of higher titered material may allow one to separate antigenic differences which could be responsible for conversion of the immunologic capacity of the virus strain after laboratory adaptation. Such immunologic conversions of virus strains may have meaning in attempting to describe mechanisms of cellular immunity, the production of spontaneous tumor regression and to hypersensitivity reactions accompanying tumor production and lympho-proliferative disease.

The occurrence of transformation in cells with infectious or with endogenous C-type viruses and herpesviruses may provide a system to study the possible interactions of DNA and RNA viruses implicated in oncogenesis. The systems which we have noted above (in guinea pigs and hamsters) are models which could be used to detect and study these possible interactions.

Serial No. NIAID 71A
1. Viral Oncology
2. Viral Diseases
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies of the Small, Highly Resistant DNA Containing Viruses Belonging to the Family Parvoviridae.

Previous Serial Number: SAME.

Principal Investigator: Dr. M. David Hoggan.

Other Investigators: Gunter F. Thomas.

Cooperating Units: Dr. F. Brent Johnson, Brigham Young University, Provo, Utah; Dr. Kenneth I. Berns, Johns Hopkins School of Medicine, Baltimore, Maryland.

Man Years:

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	35/12	0	0	25/12
Professional:	12/12	0	0	12/12
Others:	23/12	0	0	23/12

Project Description:

Objectives: To study the biological, immunological, biophysical and biochemical characteristics of the parvoviruses in order to further our understanding of the basic mechanisms of parvovirus replication and persistence, thus gaining an insight to their role in the diseases of various hosts including man.

Methods Employed: Various parvoviruses from widely divergent phylogenetic hosts are grown in their homologous host cells with various radioactive labels. The labeled virus is purified by treatment with various enzymes and isopycnic centrifugation in CsCl gradients. After treatment with appropriate antiserum to further reduce cell and helper virus contamination, the virus is rebanded in CsCl.

The viruses are disrupted then separated into their constituent polypeptides by polyacrylamide gel electrophoresis. The individual polypeptides are then characterised and antisera prepared in guinea pigs. As new parvoviruses are isolated they are purified and specific antiserum prepared in laboratory animals. Because some of the isolates grow so poorly it has been helpful to

use immune-electronmicroscopy for some of our comparative studies.

As previously reported the technique of establishing AAV-carrier cultures in the absence of helper provides a model for studying their persistence in nature. Such occult "lysogenic" cultures can be induced to produce virus, as measured by complement fixation, immunoflourescence (FA) and electronmicroscopy, by appropriate challenge with helper adenovirus. DNA/DNA reassociation kinetics using hydroxyappatite for the detection of single stranded DNA are used for detecting AAV-DNA in the "lysogenic" carrier cultures.

Major Findings: Previous studies have shown that the polypeptides of AAV-3 were not only immunogenic in experimental animals but that such antibody was specific for each of the earlier described polypeptides in vitro and further, the polypeptide antisera did not react with whole AAV virions.

On the other hand, such sera could be used to follow the development of the polypeptides in the host cell using FA. Similar polypeptide sera have now been prepared against AAV-1 and the non-helper dependent bovine parvovirus (Haden). Again the new sera do not neutralize virus but can be used in CF, FA and immunodifusion tests. The AAV-1 polypeptide sera do cross react with the other AAV-2 and 3 polypeptide antigens at 1/8 titer but do not cross react with the Haden virus. Conversely the Haden polypeptide sera do not cross react with AAV polypeptides. Again the polypeptide antigens were very unstable as compared to the protein in the intact virion. They were extremely susceptible to heat (losing antigenicity after 48 hours at 37°C and all reactivity in 10 minutes at 60°C). They were also subject to loss of activity by simple freezing and thawing (CF titer reduced 2-4 fold each time they were frozen and thawed). As had been shown with AAV-3H the polypeptide antigens of AAV-1 and Haden virus can be detected 2-3 hours earlier than the antigens associated with the whole virions. The three specific polypeptides reported earlier for AAV-3 and Haden viruses were not modified in different hosts as we had reported earlier for MVM polypeptides. The kinetics of parvovirus polypeptide antibody formation in experimental animals was somewhat similar for each parvovirus tested, i.e., low titers of antibody against the major polypeptide VP1 could be detected by 20 days after a single injection and VP2 and VP3 antibody could be detected in the Haden virus system 10-15 days after a single booster shot. It required a second booster for detection of VP2 and VP3 antibodies directed against polypeptides of AAV-1 and 2. Attempts to find polypeptide antibody in the sera of humans who had large quantities of CF and neutralizing antibody against whole virions were negative.

Using the immune electron microscopy and complement fixation tests it has been determined that an AAV isolate from dogs [Domoto and Yangawa (1964)] was totally unrelated to any of the known strains of AAV reported from man and monkeys. These results are contrasted with those of Onuma and Yanagawa (1972) who reported that the canine AAV (CAAV) cross reacted with AAV-3 and that 30% of the dog population in Japan contained CAAV-antibodies. We found no cross reactivity of the CAAV with any of the three strains of AAV-3 which we have isolated, and preliminary experiments on dog sera collected in the U.S. show some reactivity against CAAV but little or none vs. AAV-3. In our hands the

CAAV appears to be quite distinct from any of the other known AAV's tested.

Work is still in progress on the AAV-carrier cultures. They have continued to carry the virus genome after an additional 40-50 cell transfers. Some clones of the carrier cells appear to carry only part of the AAV genome. The carrier or control cells do not appear to carry even part of any of the helper virus genomes. The AAV-1 carrier clones which have been shown to produce large amounts of virus (130-180 $TCID_{50}$'s per cell) when challenged with adenovirus helper, carry approximately one AAV-genome for each host cell genome (0.7-0.9 copies per cell).

Because of rapid increase in the number of new parvoviruses isolated during the last few years with their economic impact in the veterinary field and the recent reports implicating parvovirus like agents in human disease (acute non-bacterial gastroenteritis and hepatitis type A) a two day workshop was organized and held at NIH with M. David Hoggan as Chairman. Administrative and financial support was supplied by the Research Resources Branch under the direction of Dr. John Nutter. The workshop brought together over 80 workers from throughout the world to hear 20 excellent presentations which reviewed and brought up to date the current knowledge in the area. There was much stimulating discussion which is being reviewed along with the formal presentation and will be published in Journal of Infectious Diseases early in FY 75.

Significance to Biomedical Research and the Program of the Institute: The parvoviruses continue to increase in number with a parvovirus-like agent recently being shown to be involved in infectious hepatitis in man (Hepatitis type A). Three new serological types of AAV have been described and new parvovirus candidates have been isolated from bovine diarrhea and abortion. A parvovirus-like particle has now been purified from the tissues of animals suffering from the immune complex disease known as Aleutian mink disease. In the laboratory, parvoviruses are the most teratogenic of all known animal viruses yet frequently appear as well adapted parasites in nature. For example, they can exist in a "lysogenic" provirus-like state in a mother who then passes the genome vertically to her offspring with no overt evidence of disease. They are also well suited for survival outside their normal hosts, since they are among the most resistant viruses to chemical and physical agents known. Thus it appears that the parvoviruses, although they are among some of the smallest infectious agents known (being 20-30 nm in diameter containing about 1.6×10^6 Daltons of DNA), are some of the most adaptable and versatile. Our comparative studies have helped to pinpoint the similarities and differences between the various members of the group resulting in better isolation and purification techniques. Our studies of their proteins have shown that many of the basic replicative process in different members of the group are quite similar. Besides increasing our knowledge of the mechanism of surivival in nature our studies of the parvovirus "carrier" state may unlock the genetic map of the AAV's as we isolate more clones of carrier cells which contain different amounts of the virus genome.

Proposed Course of the Project: Recent developments in the field of slab acrylamide gel electrophoresis in which it is possible to resolve many more

proteins and make direct comparison in the same gel will now allow us to do kinetic studies on polypeptide development on whole infected cell extracts. We should thus be able to elucidate more about the interrelationship of helper, host, and parvovirus protein synthesis.

Combining this technique with the use of our polypeptide sera should allow us to define the function of each polypeptide and relate it to the overall replicative process.

Our studies of the carrier state will continue and we hope to define the mechanism of persistence and whether the virus genome is integrated into the host DNA.

We shall continue the isolation of more clones of carrier cells searching for those carrying different amounts of the virus genome, which would provide the tools for mapping the viral genome.

We will also continue our comparative and epidemiological studies of parvoviruses to better define their relationship to disease.

Honors and Awards:

Chairman, NIAID RRB workshop on Parvoviruses.

Member parvovirus study group of international committee on Nomenclature of viruses.

Member WHO/FAO parvovirus committee.

Invited Seminars:

Department of Microbiology, Howard University School of Medicine, Washington, D. C.

Department of Microbiology, University of Cincinnati Medical School, Cincinnati, Ohio.

Department of Bacteriology, University of Utah, School of Medicine, Salt Lake City, Utah.

Department of Biology, Utah State University, Logan, Utah.

Department of Molecular Biology, University of California, Berkeley, California.

Department of Microbiology, Brigham Young University, Provo, Utah.

LI
LM
LBV
LMI
IPD
LSD

Publications:

Hoggan, M. David and Thomas, Gunter F.: Australia antigen. In Dalton Albert J. and Haguenau, Francoise (Eds): Ultrastructure of Animal Viruses and Bacteriophages, New York, 1973, Vol. 5, pp. 375-390.

Hoggan, M.D., Thomas, G.F. and Johnson, F.B.: Continuous "carriage" of adenovirus-associated virus genome in cell culture in the absence of helper virus. In Silvestri, L.G. (Ed.) Possible Episomes in Eukaryotes, Amsterdam, North Holland Publishing Co., 1973, pp. 243-249.

Croft, Garth F., Hoggan, M. David and Johnson, F. Brent: Production and reactivity of immune sera specific for HADEN virus polypeptide antigens, J. Virol. 13: 608-613, 1974.

Thomas, G.F. and Hoggan, M.D.: Immune Electron Microscopy (IEM) of adeno-associated virus. In Aruneaux, C.L. (Ed.) Proceedings of Electron Microscopy Society of America 32nd Annual Meeting, 1974, Claitors Publishing Division, Baton Rouge, Louisiana (In Press).

Serial No. NIAID/NCI 71 D
1. Viral Diseases
2. Viral Oncology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies of Mouse Leukemia Viruses

Previous Serial Number: Same

Principal Investigators: Dr. Janet W. Hartley, LVD, NIAID
Dr. Wallace P. Rowe, LVD, NIAID

Other Investigators: Dr. Sisir K. Chattopadhyay, LVD, NIAID
Dr. Bernice Moll, LVD, NIAID (to February 1974)
Dr. Marshall D. Sklar, LVD, NIAID
Dr. Natalie M. Teich, NCI (to September 1973)

Cooperating Units: Inside NIH:

Dr. Richard Asofsky, LMI, NIAID
Dr. Arthur S. Levine, DCT, NCI
Dr. Shigeko Nomura, VLL, NCI
Dr. Wade Parks, VC, NCI
Dr. Michael Potter, LCB, NCI
Dr. Edward Scolnick, VLL, NCI

Outside NIH:

Dr. Edward A. Boyse, Sloan Kettering Institute for Cancer Research, New York, New York
Dr. Theodore Bremner, Howard University, Washington, D.C.
Dr. Leonard Herzenberg, Stanford University Medical School, Stanford, California
Dr. H. Ikeda, Sloan Kettering Institute for Cancer Research, New York, New York
Dr. Frank Lilly, Albert Einstein College of Medicine, New York, New York
Dr. Douglas R. Lowy, Yale University School of Medicine, New Haven, Connecticut
Dr. Michael N. Oxman, Children's Hospital Medical Center, Boston, Massachusetts
Dr. John C. Parker, Microbiological Associates, Inc., Bethesda, Maryland
Dr. Paula Pitha, Johns Hopkins University, Baltimore, Maryland
Dr. E. Stockert, Sloan Kettering Institute for Cancer Research, New York, New York
Dr. James Watson, Salk Institute, La Jolla, California

LI
LM
LBV
LMI
LPD
LSD

Man Years

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	35/12	2/12	20/12	57/12
Professional:	70/12	35/12		105/12
Other:	105/12	37/12	20/12	162/12

Objectives:

To utilize sensitive procedures for genome detection and isolation and quantitation of infectious virus in studying the natural history of murine leukemia virus (MuLV) infection.

Methods employed:

Tissue culture procedures, using plaque and transformation assays; tests for antigen induction by complement fixation and fluorescent antibody procedures; reverse transcriptase assays. Mouse inbreeding, to select for specific virus inducing or virus related genes. Starch-gel electrophoresis for assays for marker isoenzyme loci. Nucleic acid hybridization techniques.

Major findings:

Genetics of MuLV infection: The high leukemia incidence, high virus mouse strain C3H/Fg has been shown to possess three independently segregating virus inducing loci. One of these loci is linked to the c locus and is, therefore, located on Linkage Group I, like the previously reported AkV_1 locus. However, the C3H/Fg locus is significantly closer to c than is AkV_1 and the gene order is different.

The high virus strain C58 also gives segregation ratios indicating 3 or possibly 4 virus-inducing loci. To date, progeny testing has not indicated that any of these loci is on Linkage Group I. Thus, the comparison of the 3 high virus strains, AKR, C3H/Fg, and C58, indicates that the virus-inducing loci are not allelic sites in the genome, but more likely represent random insertions of MuLV genetic material into the genome of the mouse.

In cooperation with the Sloan Kettering group, a large study has been carried out to clarify the relationship of the AKR virus-inducing genes to the expression of the virus-specific antigens gs and G_{IX}, the main objective being to determine if these antigens occur as a result of spread of infectious virus, or if they are induced by separate loci. The results of the study indicate that both hypotheses are true. The two virus-inducing loci induce gs and G_{IX}, but there is an additional locus which induces gs and G_{IX} in the absence of infectious virus.

Biochemical studies of MuLV infection: By comparing the nucleic acid hybridization of a single-stranded viral DNA probe, synthesized in vitro from AKR MuLV RNA, with cellular DNA from AKR mice (high leukemia, high virus) and NIH Swiss mice (low leukemia, negative for infectious mouse-tropic MuLV), it has been possible to demonstrate differences in the virus-specific sequences

represented in these two strains. A lower maximum hybridization of the viral DNA probe with the DNA from NIH Swiss cells indicated that this DNA lacks some of the viral sequences represented by the probe. In addition, Wetmur-Davidson plotting of the reassociation kinetics data revealed that while the AKR cellular DNA contains two sets of virus-specific sequences, one with 8-10 copies and one with 4 copies per haploid genome, NIH Swiss cell DNA contains only one set containing about 14 copies per genome. Further studies have shown that C3H/Fg cell DNA gives results identical to AKR, while cellular DNA from two other "virus-negative" strains, C57L and 129/J, gives reassociation kinetics similar to NIH Swiss. Intermediate strains such as DBA and BALB/c give maximal hybridization values somewhat lower than AKR but higher than NIH Swiss.

IdU induction: No significant differences in efficiency of MuLV induction by 5-iododeoxyuridine (IdU) were found in tests of embryo cells from G_{IX}^+ and G_{IX}^- lines of C57Br and C57Bl/6, or from several BALB/c X C57Bl/6 congenic lines which differ in sensitivity to mineral oil induction of plasmacytomas.

The endogenous mouse-tropic MuLV inducible by pyrimidine analogs in embryo cell cultures of various strains of inbred mice have all been reported to be N-tropic regardless of the tropism of virus isolated from post-natal animals. It has now been found that although embryo cells from the congenic strain B10.BR (a B-type mouse which produces predominantly B-tropic virus in vivo) always produce N-tropic virus following induction by IdU, cells from several embryo pools tested also produced B-tropic virus. Both N- and B-tropic viruses were inducible from two clonal lines derived from a virus-negative continuous cell line extablished from a B10.BR embryo culture.

Pathogenicity of Abelson virus: Abelson virus preparations, which appear to consist of a mixture of Moloney leukemia virus and a lymphosarcoma genome in a Moloney LV envelope, were found to induce plasmacytomas in pristane-primed BALB/c mice with an incubation period as short as three weeks. Most of the primary plasmacytomas produced myelona protein, IgA or IgG.

Preliminary data suggest that Abelson virus can transform B lymphocytes in vitro. Infection of splenic lymphocytes in short term tissue culture, followed by transplantation into histocompatible mice with a karyologic marker different from that of the donor cells, has led to formation of lymphosarcomas with donor karyotype.

MuLV with heterologous species host range: Efforts have been made to develop sensitive isolation techniques and quantitation procedures for the xenotropic (X-tropic) class of MuLV. Isolation attempts from BALB/c, C57Br, C57Bl/6, and C57/L cells derived from embryos or young adult kidney-spleen have been uniformly more strongly positive after IdU treatment. IdU treatment of NZB cell lines, which are spontaneously positive for particles and RNA-dependent DNA polymerase, results in a virus population which can readily replicate in human (RD) or rabbit (SIRC) cells; in contrast, the virus from untreated cultures replicates much less efficiently. The rabbit cornea cell line SIRC, described by Todaro et al. as highly sensitive to X-tropic virus as assayed by RTP assay, is also

a satisfactory system for assays by fluorescent antigen induction.

Mouse-tropic MuLV interfere completely with each other regardless of serologic type or host range. Using MSV pseudotypes of mouse- and X-tropic MuLV isolates for virus interference assays on NRK cells, it was found that the X-tropic viruses are not significantly inhibited in cells solidly resistant to mouse-tropic viruses.

MuLV of feral origin: It was noted by us and others that MuLV isolates from wild mouse populations were capable of efficient growth in both mouse and heterologous species host cells, in contrast to the restricted range of X-tropic and mouse-tropic viruses. In an attempt to test for distinct host-range components in these isolates, purification by limiting dilution titration in tissue culture are in progress for two isolates from different sources. Preliminary results indicate that a stable mouse-tropic component is present which does not replicate in RD or SIRC cells; it has not yet been possible to dissect out a population which can grow in heterologous species cells but not, at least to some extent, in mouse cells.

Significance to Biomedical Research and the Program of the Institute:

Understanding the genetics of transmission of information for infectious virus and virus-specified products and control of virus spread will provide basic parts of the complex picture of tumorigenesis in mice. Approaches which contribute to the understanding of MuLV genetics and which may eventually permit correlations with development of particular types of tumors include the precise chromosomal localization of V genes, studies of the stability of identifiable viral gene integration sites, the indication that integration sites are non-allelic in various strains of mice, and studies of the relationship of Linkage Group I to viral gene integration.

The ability to distinguish between the viral sequences in DNA from mouse strains which differ in MuLV biology makes feasible the molecular study of virus expression in a variety of mouse strains and in genetic crosses segregating for various expressions of MuLV infection.

Studies of the biology of MuLV infection at the levels of both natural history and molecular biology now require investigating the role of the X-tropic virus genome. The isolates from feral mice provide an opportunity to study a naturally occurring virus population which may represent a complex of homozygous and recombinant particles.

The induction of plasmacytomas by Abelson virus and development of an *in vitro* lymphocytic transformation system represent major developments in immunology and leukemogenesis.

Proposed Course:

Studies will continue on the genetic relationship of various markers of MuLV information or control and on the localization of the unmapped virus-inducing genes in AKR, C3H/Fg, and C58 mice.

Nucleic acid hybridization studies will continue to compare the homology of cellular DNA from mouse strains of differing characteristics and viral probes prepared from AKR MuLV and other mouse cell-tropic MuLV, and X-tropic isolates.

Studies on IdU induction will particularly emphasize X-tropic virus and means of directly quantitating the number of cells induced. Serologic comparisons of X-tropic viruses from various sources will be carried out, and studies will be continued to clarify the host range and serologic properties of MuLV from wild mice.

Intensive efforts will be made to define the optimal conditions for obtaining in vitro transformation of lymphocytes, to develop in vitro methods for assay of the transformation, and to obtain tumors from cells committed to synthesis of particular antibodies.

Honors and Awards:

Awards:

Dr. Wallace P. Rowe -	USPHS Distinguished Service Medal Howard Taylor Ricketts Award for 1974

Honors:

Invited Lectures:

Dr. Wallace P. Rowe -	Symposium lecture at FASEB Meeting, Atlantic City, New Jersey Symposium on The Biology of Cancer, University of Michigan Medical Center, Ann Arbor, Michigan Course in Diagnostic Virology, The W. Alton Jones Cell Science Center, Lake Placid, New York Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland International Congress of Genetics, Berkeley, California St. Jude Children's Research Hospital, Memphis, Tennessee University of Pennsylvania, Philadelphia, Pennsylvania

Dr. Sisir Chattopadhyay - Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Committees:

Dr. Wallace P. Rowe - Ad Hoc Committee to Evaluate the Virus Cancer Program of the NCI
Two Ad Hoc Committees to Evaluate Appointment of Faculty - Harvard
University of Washington
NIH Biohazard Committee

Dr. Janet W. Hartley - Virology and Cell Biology Advisory Committee, American Cancer Society
NCI Program Project Grant Site Visit Committee to Sloan-Kettering Institute

Editorships:

Dr. Wallace P. Rowe - Editor, ARCHIV FUR DIE GESAMTE VIRUSFORSCHUNG
Editorial Board - INFECTION AND IMMUNITY
INTERVIROLOGY
BBA REVIEWS ON CANCER

Dr. Janet W. Hartley - Editorial Board - JOURNAL OF IMMUNOLOGY

Publications:

Rowe, W.P., Humphrey, J.B., and Lilly, F.: A major genetic locus affecting resistance to infection with murine leukemia viruses. III. Assignment of the Fv-1 locus to linkage group VIII of the mouse. J. Exp. Med. 137:850-853, 1973.

Ikeda, H., Stockert, E., Rowe, W.P., Boyse, E.A., Lilly, F., Sato, H., Jacobs, S., and Old, L.J.: Relation of chromosome 4 (linkage group VIII) to MuLV-associated antigens of AKR mice. J. Exp. Med. 137:1103-1107, 1973.

Chattopadhyay, Sisir K., Lowy, Douglas R., Teich, Natalie M., Levine, Arthur S., and Rowe, Wallace P.: Evidence that the AKR murine-leukemia-virus genome is complete in DNA of the high-virus AKR mouse and incomplete in the DNA of the "virus-negative" NIH mouse. Proc. Natl. Acad. Sci. USA 71:167-171, 1974.

Gardner, M.B., J.E. Officer, R.W. Rongey, H.P. Charman, Janet W. Hartley, J.D. Estes, and R.J. Huebner: C-type RNA tumor virus in wild house mice (Mus musculus). In Unifying Concepts of Leukemia, Bibl. haemat., No. 39, ed. by R.M. Dutcher and L. Chieco-Bianchi (Karger, Basel) 1973, pp. 335-344.

Levy, Jay A., Hartley, Janet W., Rowe, Wallace P., and Huebner, Robert J.: Studies of FBJ osteosarcoma virus in tissue culture. I. Biologic characteristics of the "C"-type viruses. J. Natl. Cancer Inst. 51:525-539, 1973.

Potter, Michael, Sklar, Marshall D., and Rowe, Wallace P.: Rapid viral induction of plasmacytomas in pristane-primed BALB/c mice. Science 182:592-594, 1973.

Rowe, Wallace P.: Genetic factors in the natural history of murine leukemia virus infection: G. H. A. Clowes Memorial Lecture. Cancer Res. 33:3061-3068, 1973.

Serial No. NIAID 71E
1. Viral Diseases
2. Viral Oncology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies of Viral Antigens in Virus-induced Tumors.

Previous Serial Number: SAME.

Principal Investigators: Drs. Andrew M. Lewis, Jr. and Wallace P. Rowe.

Other Investigators: None.

Cooperating Units: Drs. Arthur Levine, Lowell Schnipper, Cephas Patch, and James Breeden (Medicine Branch, NCI); Michael Oxman, Myron Levin (Harvard Medical School); Thomas J. Kelly, Jr., (Johns Hopkins University); Clyde S. Crumpacker, II, (Channing Laboratory, Boston City Hospital); Lenneart Philipson (University of Uppsala, Sweden); Satvir Tevethia, (Tufts University); Sherman Weissman, (Yale University).

Man Years:

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	31/12	12/12	0	43/12
Professional:	13/12	0	0	13/12
Other:	18/12	12/12	0	30/12

Project Description:

Objectives: To use the adenovirus-SV40 hybrid viruses as tools to study the genetics of DNA tumor viruses; to define the role of viral genes and antigens in the oncogenic process; to study the biology of adenovirus-SV40 hybrid viruses.

Methods Employed: Tissue culture, fluorescent antibody, complement fixation, serologic epidemiology, and nucleic acid homology techniques.

Major Findings - Studies on nondefective adenovirus-SV40 hybrid viruses: The properties of the five nondefective adenovirus 2-SV40 hybrids which we have isolated and characterized were described in detail in the 1972-1973 project report. These hybrid viruses are genetic recombinants between adenovirus 2 and SV40 which contain between 6 and 48% of the SV40 genome inserted at the same location in the adenovirus 2 genome. Three of these hybrids ($Ad2^{+}ND_1$

$Ad2^+ND_2$ and $Ad2^+ND_4$) induce SV40 U; U, TSTA; or U, TSTA, and T antigens respectively. The ability of these hybrids to induce more than 1 SV40-specific antigen is associated with the incorporation of increasingly larger segments of the SV40 genome into adenovirus 2 DNA. Thus, $Ad2^+ND_1$ which induces U antigen contains 17% of the SV40 genome while $Ad2^+ND_4$ which induces SV40 U, TSTA and T antigens contains 48% of the SV40 genome. By heteroduplex mapping techniques the regions responsible for inducing these SV40 functions have been located on the SV40 genome. In addition, using both nucleic acid hybridization and heteroduplex mapping, the SV40 DNA segments in each of the five nondefective hybrids have been mapped on the SV40 genome with respect to the cleavage sites of SV40 DNA produced by restriction endonucleases from Hemophilus influenzae and Hemophilus parainfluenzae.

1. Transcription of SV40 DNA Segments During Lytic Infection: Others have shown that cells lytically infected with SV40 synthesize viral RNA in two phases, i.e., early SV40 genes are transcribed before SV40 DNA replication begins while both early and late genes are transcribed after the onset of viral DNA replication. Using separated strands of SV40 DNA it has also been shown that the early SV40 genes are on the minus strand while the late SV40 genes are on the plus strand. Since the integration of SV40 DNA into the genome of transformed cells resulted in transcription of the minus strand of SV40 DNA, we tested RNA from cells lytically infected with each of the non-defective hybrids. Like SV40 RNA from transformed cells, the SV40 RNA from nondefective hybrid infected cells reacted only with the minus strand of SV40 DNA. In addition, the RNA's from the nondefective hybrids reacted extensively with the minus strand of the G fragment (produced by digestion of SV40 DNA with hemophilus influenzae restriction enzyme) of SV40 DNA. From the position of the G fragment in the SV40 genome, we concluded that anti-late SV40 sequences are also transcribed during lytic infection by the nondefective hybrids. None of the hybrid RNA's reacted with the plus strand of SV40 DNA even though other experiments (described below) indicate that each of the SV40 DNA segments in the nondefective hybrids contains roughly equal quantities of early and late SV40 DNA sequences.

2. Organization of the Early SV40 Genome: To determine the relationships of early and late SV40 DNA sequences in the SV40 segments contained in the non-defective hybrid genomes, the strands of the hybrid DNAs were separated using synthetic polyribonucleotides and reacted with complementary SV40 RNA, SV40 RNA which is synthesized *in vitro* from Form I SV40 DNA using *E. coli* RNA polymerase and SV40 RNA from cells which had been infected for 48 hours (early + late SV40 RNA). Previous studies showed that the SV40 DNA segment in the $Ad2^+ND_1$ hybrid (representing about 17% of the SV40 genome) contains both early and late SV40 DNA sequences. Since transcription of early and late SV40 DNA proceeds on opposite strands of the SV40 genome, finding both early and late SV40 sequences in the $Ad2^+ND_1$ genome suggested the presence of a transcription control region in this segment of SV40 DNA. In addition, these studies on the separated strands of $Ad2^+ND_1$ DNA showed that early SV40 DNA sequences were located on the light strand of the hybrid DNA while the late sequences were located on the heavy strand. The ratio of ^3H RNA counts

of early + late SV40 RNA which bound to the light $Ad2^{+}ND_1$ DNA strand to the ^3H RNA counts of early + late SV40 RNA which bound to the heavy DNA strand was about 1. Since this SV40 DNA segment represented only 17% of the SV40 genome it seemed reasonable to assume that the amount of early + late SV40 RNA binding to the light strand should become progressively larger in those hybrid SV40 DNA segments which contained larger amounts of early SV40 DNA. Therefore, the ratio of early + late SV40 RNA binding to light versus heavy strands of the larger segments of SV40 DNA should be greater than 1. By examining the binding of early + late SV40 RNA with the separated strands of DNA from the other nondefective hybrids, it should have been possible to determine if a second transcription control region existed in the larger segment of SV40 DNA in the $Ad2^{+}ND_4$ genome which represented 48% of the wild-type SV40 genome. When the separated strands from the hybrids containing the largest SV40 DNA segments ($Ad2^{+}ND_2$, $Ad2^{+}ND_4$, $Ad2^{+}ND_5$) were reacted with early + late SV40 RNA equal amounts of this RNA reacted with each strand. This finding suggested that the 48% of the SV40 genome which contained the early SV40 region contained equal amounts of early and late SV40 DNA sequences. This concept disagrees with the current model proposed for the organization of the early regions of the SV40 genome. If confirmed by additional studies it will show that the early SV40 genome and early SV40 protein are much smaller than other studies suggest.

3. Effect of Interferon on SV40 Antigen and RNA Production: In this laboratory in 1967 Dr. M. N. Oxman noted that the induction of SV40 T antigen by the defective Ad2 and Ad7 SV40 hybrid populations was markedly resistant to interferon compared with the interferon sensitivity of T antigen induction during lytic infection by SV40. As the induction of Ad T antigens during lytic adenovirus infection demonstrated the same degree of resistance as SV40 T antigen induction by the hybrid virions, Oxman speculated that transcription of the SV40 segment in the hybrid virus DNA was under the control of the adenovirus genome rather than under the control of the incorporated SV40 DNA segment.

Following up these experiments in a collaborative study, Drs. Oxman, Levin and myself compared the interferon sensitivity of SV40 T antigen induction by the nondefective hybrid virus, $Ad2^{+}ND_4$, with the interferon sensitivity of wild type SV40. These experiments demonstrated that SV40 T antigen induction by this hybrid was resistant to doses of interferon which drastically reduce SV40 T antigen induction by SV40. Ad2 T antigen induction by $Ad2^{+}ND_4$ showed nearly identical resistance to interferon as its SV40 T antigen induction.

If the hypothesis that transcription of the SV40 DNA segment in the hybrid genome was initiated in the adenovirus region was correct then RNA molecules containing both Ad2 and SV40 RNA sequences should be detectable in $Ad2^{+}ND_4$ infected cells. By first reacting radiolabeled RNA from cells infected with $Ad2^{+}ND_4$ with Ad2 DNA, eluting the bound RNA, and rehybridizing it with SV40 DNA_1 7 to 11% of the SV40 specific RNA in $Ad2^{+}ND_4$ infected cells which had been initially bound to Ad2 DNA was recovered on SV40 DNA filters. By comparing the recovery of SV40 specific RNA when the first filter contained Ad2 DNA with the percent recovered when the first filter contained SV40 DNA

it appears that at least 27% of the SV40-specific RNA synthesized in $Ad2^{+}ND_4$ infected cells is covalently linked to Ad2 RNA. Knowing that the direction of transcription of the early SV40 genome proceeds from T towards U and that the early SV40 RNA sequences in the $Ad2^{+}ND_4$ hybrid are located on the light strand of Ad2 DNA suggests that transcription of the $Ad2^{+}ND_4$ genome is initiated upstream from the SV40 T antigen region in the adenovirus portion of the hybrid DNA molecule. This would result in the synthesis of a polycystronic RNA molecule containing Ad2 sequences on the 5' end and establish the direction of transcription of Ad2 DNA.

In another series of experiments, Oxman and Levin showed that not only was SV40 T antigen induction sensitive to interferon but in addition there was a corresponding reduction in the amount of early SV40 RNA in interferon treated cells. This finding suggested that interferon was acting as an inhibitor of transcription in SV40 infected cells. We have followed up this observation by comparing the effects of inhibitors of DNA synthesis (Cytosine arabinoside and 5-Fluorodeoxyuridine) and interferon on the production of virus specific RNA in cells infected by SV40 and the nondefective hybrid $Ad2^{+}ND_1$. These experiments indicated that inhibitors of DNA synthesis and 200 U/ml of interferon reduced the production of virus specific RNA about 100-fold in SV40 infected cells. In $Ad2^{+}ND_1$ infected cells inhibitors of DNA synthesis produced a 15-20 fold reduction in SV40 RNA while the same dose of interferon affected only a 2-3 fold reduction in SV40 RNA synthesis. These findings agree with the observation that SV40 U antigen synthesis is partially sensitive to inhibitors of DNA synthesis in $Ad2^{+}ND_1$ infected cells. In contrast to the dramatic effect interferon has on viral RNA synthesis in SV40 infected cells it has little effect on SV40 RNA synthesis in cells infected by this nondefective hybrid virus. Unfortunately, these studies have been suspended for the past 14 months due to lack of facilities for safe propagation of the nondefective viruses.

4. Transformation of Hamster Cells by Ad2 and the Nondefective Hybrids: Having established that the nondefective Ad2-SV40 hybrids contain varying segments of the 48% of the SV40 genome which is expressed in SV40 induced tumors and transformed cells, we were interested in determining the effect of these SV40 DNA segments on the ability of Ad2 to induce tumors and transform cells in tissue culture. During subsequent testing nonhybrid Ad2 and the nondefective hybrids failed to produce tumors when injected into newborn hamsters. However, each of these agents transformed hamster kidney cells *in vitro* and the transformed cell lines produced tumors when injected into suckling hamsters. Foci of cells transformed by the hybrids could not be distinguished from foci of cells transformed by nonhybrid Ad2. Histopathologically, tumors induced by the nondefective hybrid transformed lines were of the adenovirus type with no areas of SV40 type fibrosarcoma. All of the cell lines studied contained Ad2 T antigen and Ad2 RNA. Only the $B55HK_1$ line contained the three early SV40 antigens, U, TSTA, and T. The ND_4HK_1 line contained SV40 T and TSTA antigens (the latter antigen being detected only after 48-49 tissue culture passages) and SV40 RNA. The ND_2HK_2 line contained SV40 RNA but no detectable SV40 antigens; the other hybrid transformed lines contained no SV40 RNA. The transformation of hamster cells by the nondefective

Ad2-SV40 hybrids seemed to be caused by the Ad2 genome and could not be attributed to the regions of the SV40 genome contained within the hybrids. Additionally, these results indicate that interactions between the genomes of the nondefective hybrid viruses and the hamster cells they transform are complex, and could involve the site of integration, the size of the incorporated viral genome, the transcription or translation of the viral genome, or various combinations of these processes.

To further evaluate the effect of early regions of the SV40 genome on transformation of hamster cells by Ad2, assays for measuring the efficiency of transformation of hamster cells by Ad2 have been devised. Using UV inactivated virus to retard cytopathic changes, we have found that the efficiency of transformation of 5 lots of hamster embryo cells varied between $10^{6.7}$ plaque forming units per focus (PFU/FFU) forming units to $10^{8.4}$ PFU/FFU. Having established the usefulness of such an assay, studies comparing the efficiency of transformation by UV inactivated Ad2 and several of the nondefective hybrids are underway.

Major Findings - Studies on Defective Adenovirus SV40 Hybrid Viruses: We have described two variants of the defective $Ad2^{++}$ hybrid population which differ in their ability to yield SV40 virions. The $Ad2^{++}$HEY (high efficiency yielder) produces about 10,000 times more SV40 progeny than does the $Ad2^{++}$LEY (low efficiency yielder). Both these variants induce SV40 T antigen but only $Ad2^{++}$HEY induces detectable SV40 V antigen.

1. Electronmicroscopic heteroduplex mapping: The structure of the DNA molecules in these populations was examined by electronmicroscopic heteroduplex mapping. The DNAs from both populations were heterogeneous. $Ad2^{++}$ HEY contains centrally inserted SV40 DNA accompanied by a 39% Ad2 DNA deletion. Three hybrids were noted (HEY I, HEY II, and HEY III) whose genomes differed only in their content of SV40 DNA (0.45, 1.43 and 2.39 SV40 genomes respectively). In these hybrids the SV40 insertion runs in the opposite direction from the SV40 insertion in the nondefective hybrid DNA, i.e., the early SV40 region on the minus strand of SV40 DNA is linked to the heavy strand of Ad2 DNA rather than to the light Ad2 strand. The SV40 DNA segments are inserted into the $Ad2^{++}$ LEY genome in the AT rich half of the molecule 6% from the end and are accompanied by a 11% deletion of Ad2 DNA. $Ad2^{++}$ LEY contained two hybrids (LEY-I and LEY-II) which also differed only in their content of SV40 DNA (0.03 and 1.05 SV40 genomes, respectively). The SV40 DNA inserted into the $Ad2^{++}$ LEY genome shared a common end point at 48% of the SV40 genome from the site cleaved by E. coli R1 endonucleose with SV40 DNA segment in the $Ad2^{+}ND_4$ hybrid and had the same strand alignment as the nondefective hybrids, i.e., the early SV40 region on the minus SV40 strand is joined to the light Ad2 strand. In those hybrids which contained more than one complete SV40 genome (HEY II, HEY III, LEY II) the excess SV40 DNA was shown to be organized as a tandem repetition. These data suggest that the various hybrid genomes within each population are interconvertible by recombination events which insert or excise an SV40 genome. We proposed that HEY II and HEY III yield infectious SV40 with higher efficiency than LEY II because their SV40 DNA segments contain longer tandem repetitions which would greatly increase the probability of an

intramolecular recombination event which results in excision of an SV40 genome.

2. Transcription and SV40 Antigen Induction: Hybridization competition experiments indicate that late SV40 RNA is not transcribed efficiently in $Ad2^{++}$ LEY infection in contrast to $Ad2^{++}$ HEY infection and only two SV40 antigens, T and U, are detected in Ad^{++} LEY infected cells. SV40 transplantation antigen (TSTA) is not detected in $Ad2^{++}$ LEY immunized hamsters but is detected in $Ad2^{++}$ HEY immunized hamsters. Studies with the nondefective hybrid viruses demonstrate that the SV40 TSTA maps between the templates for SV40 T and U antigens thus the failure to detect SV40 TSTA in $Ad2^{++}$ LEY immunized hamsters is surprising. If the early SV40 genes were under a single regulatory control mechanism, TSTA should be formed in amounts comparable to T and U antigens. Failure to detect TSTA in hamsters could be quantitative, i.e., the TSTA bioassay may be less sensitive than the serologic assays for SV40 T and U antigens. In $Ad2^{++}$ HEY immunized animals more TSTA might be induced as a function of increased SV40 replication. However, we have recently shown that the early SV40 templates may not be contiguous; therefore, it is possible that the three early templates are under separate controls. In that case the SV40 genome in $Ad2^{++}$ LEY hybrid virions might contain an alteration in the promotor for SV40 TSTA transcription. The finding that $Ad2^{++}$ LEY produces early SV40 RNA which competes with only 70% of early SV40 RNA supports this concept. Collaborative studies with Dr. S. Tevethia indicate that $Ad2^{++}$ LEY will induce detectable SV40 TSTA in immunized mice thus this hybrid is not absolutely defective in its ability to induce this SV40 antigen.

The failure of $Ad2^{++}$ LEY to efficiently induce the synthesis of late SV40 RNA transcripts, despite the presence in the hybrid particle of the entire SV40 genome, is similar to two other situations in which the expression of integrated SV40 DNA has been studied. In SV40-transformed cells, viral-specific RNA is transcribed primarily (and in certain lines exclusively) from the (-) strand of SV40 DNA, and therefore represents only early or anti-late SV40 templates even though late templates (+ strand) may be present. Secondly, this same pattern of transcription has been found in cells acutely infected with the nondefective Ad2-SV40 hybrid viruses, $Ad2^{+}ND_1$ - $Ad2^{+}ND_5$, where the SV40-specific transcripts are complementary only to the (-) strand of SV40 DNA despite the presence of late (+ strand) templates within the SV40 moiety of these hybrids. Thus, it seems very likely, on the basis of these three situations, that unless SV40 DNA is in a non-integrated form (as in Ad2 HEY-infected cells after excision) expression of the late genes (+ strand) is limited or absent.

Significance to Biomedical Research and the Program of the Institute: The usefulness of the nondefective Ad2-SV40 hybrids for genetic studies on SV40 and Ad2 are widely recognized. The ability of these agents to transform cells and the diverse patterns of viral antigen and RNA detected in the transformed cell indicates their potential usefulness in the elucidation of mechanisms of genetic control in animal cells.

The Institute has a vital interest in antiviral substances. The marked differences in interferons sensitivity of SV40 T antigen and RNA production

LI
LM
LBV
LMI
LSD

by non-integrated and integrated SV40 DNA offers a unique approach to understanding some of the mechanism of action of this substance.

The $Ad2^{++}$ HEY and $Ad2^{++}$ LEY hybrid populations are of particular interest because they appear to be useful models for analyzing the properties of integrated SV40 genomes. It is known that in cells transformed by SV40, the viral genome is stably integrated by covalent linkage to host cell DNA (many SV40 transformed cell lines), and that infectious SV40 can frequently be recovered from such cells by fusion with permissive cells. Among the major differences that exist between cells lytically infected and transformed by SV40 are: (1) Most newly synthesized viral DNA is not integrated in the case of lytic infection, but most viral DNA appears to be integrated in the case of transformation; (2) SV40-specific RNA is synthesized both early and late during lytic infection, but SV40 RNA from transformed cells is primarily early; (3) Early SV40 (T, U, TSTA) antigens are detectable both in lytic infection and transformation, but late SV40 V antigen is only detectable following lytic infection. Our studies indicate that these three differences are associated with lytic infection by $Ad2^{++}$ HEY and $Ad2^{++}$ LEY.

The implication of the discussions about the potential hazards posed by recombinants like the nondefective Ad2-SV40 hybrids for both biomedical research and the programs of the Institute were discussed in the last annual report. In that report we noted that the fundamental issue in these deliberations was whether the rewards outweighed the risks inherent in the use of such mutants. The demonstrated and potential usefulness of these agents in understanding biological processes is outlined in this report. In the fall of 1973 the results of discussions concerning the risks involved in work with the nondefective hybrids were compiled in a document (The Memorandum of Understanding and Agreement) which outlines these risks and establishes requirements which all investigators are obligated to meet before receiving or working with these agents. This document attempts for the first time to establish a mechanism for dealing with laboratory created agents which can be considered potentially hazardous. The only workable mechanism for enforcing such requirements is the concern and voluntary compliance of the individual investigator. The generation of this document on studies of nondefective hybrids in NIAID required a 14 month moratorium on the propagation of these agents. Due to the priority given this work by NIAID no containment facilities will be available in this Institute for another 18 months to 2 years.

The implications of a 2 1/2 to 3 year delay on work with nondefective hybrid viruses in this Institute are already affecting the willingness of investigators in other institutions who are interested in working with recombinants to cooperate with the Memorandum of Understanding and Agreement mechanism. In addition, the threat of such delays will likely affect the judgement of these investigators when they consider the extent to which they involve administrative personnel in future problems of a similar nature.

Proposed Course of the Project: The 13 papers published on work with nondefective hybrid viruses in 1973-1974 culminated seven years of intense work. Due to the lack of containment facilities we have been unable to propagate the

nondefective hybrid since Feb. 1973. The momentum generated since these studies were initiated in 1967 has been severely reduced. The extent of this slow down is apparent when the work which has been curtailed is reviewed. Studies on the sequencing of the SV40 DNA segments in the nondefective hybrids have been suspended. Studies on the mechanism of action of interferon on viral RNA transcription have been suspended. Studies on the transcription of the nondefective hybrid genome during lytic infection have been suspended. Studies attempting to deleniate the apparent instability of the B55 and $Ad2^{+}ND_4$ genome in human renal cells have been suspended. No attempts have been made to isolate the $Ad2^{+}ND_{4\ del}$ hybrid discovered during heteroduplex mapping studies 18 months ago. No attempts have been made to develop techniques to isolate additional hybrids. Studies to determine if the nondefective SV40 DNA segments isolated by E. coli RI restriction endonuclease digestion would transform cells have been postponed. Without adequate containment facilities and technical help to allow resumption of biological studies in the very near future the initiatives provided by the isolation and characterization of these agents in NIAID will be lost.

Until proper facilities are available studies will necessarily be restricted to completing studies initiated prior to February 1973, transformation assays using UV inactivated viruses and the isolation and characterization of transformed cell lines.

Honors and Awards:

Awards:

Dr. Andrew M. Lewis	PHS Commendation Award

Honors:

Invited Lectures:

Dr. Andrew M. Lewis	Gordon Research Conference on Animal Cells and Viruses, Meridan, New Hampshire 1974 Spring Meeting, Institute of Genetics, University of Cologne, Cologne, Germany Cold Spring Laboratory, Cold Spring Harbor, New York

Publications:

Lewis, Andrew M., Jr., Levine, Arthur S., Crumpacker, Clyde S., Levine, Myron J., Samaha, Richard J., and Henry, Patrick H.: Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. V. Isolation of additional hybrids which differ in their simian virus 40-specific biological properties. J. Virol. 11: 655-664, 1973.

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adenovirus 2-simian virus 40 hybrid viruses. VI. Characterization of the deoxyribonucleic acid from five nondefective hybrid viruses. J. Virol. 11: 665-671, 1973.

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Kelly, Thomas J., Jr., and Lewis, Andrew M., Jr.: The use of nondefective adenovirus-simian virus 40 hybrids for mapping the simian virus 40 genome. J. Virol. 12: 643-652, 1973.

Morrow, John F., Berg, Paul, Kelly, Thomas J., Jr., and Lewis, Andrew M., Jr.: Mapping of simian virus 40 early functions on the viral chromosome. J. Virol. 12: 653-658, 1973.

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Patch, Cephas T., Lewis, Andrew M., Jr., and Levine, Arthur S.: Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. IX. Template topography in the early region of simian virus 40. J. Virol. 13: 677-689, 1974.

Lewis, Andrew M., Jr., Rabson, Alan S., and Levine, Arthur S.: Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. X. Transformation of hamster kidney cells by adenovirus 2 and the nondefective hybrid viruses. J. Virol., in press.

Lebowitz, Paul, Kelly, Jr., Thomas J., Nathans, Daniel, Lee, T.N.H., Lewis, Andrew M., Jr.: A colinear map relating the simian virus 40 (SV40 DNA segments of six adenovirus-SV40 hybrids to the DNA fragments produced by restriction endonuclease cleavage of SV40 DNA. Proc. Nat. Acad. Sci, USA 71: 441-445, 1974.

Serial No. NIAID-71-F
1. Laboratory of Viral Diseases
2. Molecular Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on Interferon and Interferon Inducers

Previous Serial Number: SAME

Principal Investigator: Dr. Hilton B. Levy

Other Investigators: Mr. Freddie Riley

Cooperating Units: Dr. George Baer, CDC, Atlanta Ga.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	36/12			36/12
Professional	12/12			12/12
Other	24/12			24/12

Project Description:

Objectives

To study the interferon-induced alterations in cells that enable these cells to develop resistance to virus. To determine how such alterations may activate the several other functions recently described for interferon. To develop the interferon system so that it may be used in therapy of virus diseases and malignancies in man.

Methods Employed

Many biochemical methods have been employed. Included, but not all inclusive, are spectrophotometry, single and double isotope tracing, gel electrophoresis, gradient centrifugation, paper and column chromatography, etc. In addition, various biological techniques have been employed, both in tissue culture

and in animals, as indicated in the sections on results. Several extensions of the intramural program have been developed in collaborative programs through the contract branch of LMAIP.

Major Findings

The previous year's report summarized our data showing that in interferon-treated cells there is a unique fraction of cellular RNA that is different from the corresponding fraction in cells not treated with interferon. This RNA fraction is nonribosomal RNA found associated with polysomes. We suggested, based on the way the fraction was prepared, that the material represents messenger RNA that was in the process of being translated, together with associated transfer RNA. The fraction from interferon-treated cells differed from normal material in being slightly larger. We suggested that interferon treated cells may be able to distinguish viral RNA from cellular messenger RNA by modifying its own cellular messenger RNA so that it is different from both normal cellular RNA and viral RNA in a characteristic that is important for recognition. Modified t-RNA under those circumstances might play a role in the recognition mechanism.

This year has established more definitively the nature of the polysome associated RNA. The material that we had tentatively called transfer RNA has been more firmly characterized; it is rich in methyl groups and since only t-RNA is so enriched this is strong evidence that the material is indeed t-RNA. Further, it sediments in sucrose gradients at 4S which is an additional characteristic of t-RNA. The material that we had called messenger RNA shows the characteristic of most messenger RNAs, namely it binds to a column of poly-d-thymidine cellulose because the RNA contains a poly A fragment. However we have not been able to confirm or rule out the possibility that the poly A size is different from normal. We have been trying to prepare larger quantities of the material in order to better characterize the fractions.

An additional piece of RNA smaller than t-RNA had been found in association with polysomes that shows the interferon-induced size changes alluded to. The nature of this polynucleotide is being investigated.

Stabilization of poly I.poly C. Previous work from this laboratory and others has shown that the poly I.poly C is a good antiviral and antitumor agent in rodents. However, it has proved disappointing in both these regards in man and in other primates.

A few years ago we showed that there is present in human serum a high level of activity of a nuclease complex that destroys poly I.poly C. Other animals with such a high level of serum nucleases also have minimal responses to poly I.poly C. It was determined that efforts through the contract mechanism would be made to develop nuclease resistant derivatives of the inducer but such efforts were not successful. Acting on an observation made a few years ago by Dr. Rice of the National Cancer Institute, we succeeded intramurally in preparing a three-way complex of poly I.poly C with low molecular weight poly-l-lysine and carboxymethylcellulose. This complex is partially resistant to hydrolysis by serum nucleases. It induces good quantities of interferon in mice, possibly more than poly I.poly C itself. More important, it induces good quantities of interferon in monkeys and in chimpanzees, which poly I.poly C did not. When the complex is given intrathecally into monkeys, 1,000 to 2,000 units of interferon per ml of cerebrospinal fluid is found, with no gross toxicity seen, Toxicity in general appears to be no greater than poly I.poly C and perhaps is less. Antigenicity has not been seen but much more extensive observations are needed before the antigenic possibilities are evaluated.

Significance to Biomedical Research and the Program of the Institute

The question of how cells determine which among the many proteins they can make will actually be synthesized, has been the subject of much investigation. The present observations suggest a new way in which such selective control can be exercised. Interferon-induced discrimination against translation of viral messenger RNA may be only the most readily detectable among several types of messenger RNAs selected against. The several newly described actions of interferon such as interactions with the immune system may be related to this broad biochemical alteration induced by interferon.

Availability of an interferon inducer that is biologically active in primates offers the possibility that it may be effective in man. By analogy the poly I.poly C poly-lysine complex might be expected to act as adjuvant for both humoral and cell mediated immunological actions. Effectiveness against virus disease and malignancies is an encouraging possibility.

Proposed Course of the Project

Continued efforts to prepare larger quantities of these altered RNA fractions will be made in order to carry out more biological and biochemical studies.

Toxicology studies on the poly I.poly C poly lysine complex are planned for the immediate future. If warranted, trials in man versus suitable virus diseases and tumors will be initiated.

Publications:

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Serial No. NIAID-71-G
1. Laboratory of Viral Diseases
2. Cellular Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Structure and Replication of Viruses

Previous Serial Number: 3MPT

Principal Investigator: Dr. C. F. T. Mattern

Other Investigators: Wendell A. Daniel, M. S.
Anna M. Kontonis, M. S.

Cooperating Units: Dr. Louis S. Diamond, LPD, NIAID, 122-G1
Dr. Joseph T. Marino, LPD, NIAID, 122-G1
Dr. Shigeko Nomura, VLL, NCI-4835
Dr. Paul Peebles, VLL, NCI-4835
Dr. Kenneth K. Takemoto, LVD, NIAID, 71-J

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	19/12			19/12
Professional	6/12			6/12
Other	13/12			13/12

Project Description:

Objectives

The major emphasis of the project falls into three areas: (1) the continued study of biological, physical and chemical properties of viruses of Entamoeba histolytica and their possible relationship to human disease. (2) We are attempting to isolate new viruses from other protozoa which are pathogenic for man. (3) Collaborative studies on the potential of murine sarcoma virus-transformed cells to revert and retransform, to produce defective C-particles, reverse transcriptase and antigen, their rescuability with MuLV, and their inducibility with IUdR.

Methods Employed

These studies involve high resolution electron microscopy of viruses and sections of infected and normal cells employing a variety of viruses and cell strains. A variety of chemical and physical techniques are utilized in the purification and study of the biological and chemical properties of viruses.

Tissue culture is employed in attempts to induce infection of vertebrate cells by known amoebal viruses and to isolate putative agents from other parasitic protozoa.

Major Findings

(1) Amebal viruses. (a) First and second clones of E. histolytica are under continued study. All clones of strain HB:3#1 so far isolated in the presence of virus antiserum have produced the polyhedral virus (V3J1) (Diamond et al). Whether or not this apparent lysogenic system involves integration of viral DNA into amoebal DNA is under continued investigation (Mattern et al.).

(b) A third morphological entity has been identified within nuclei of infected cells. It is a unique, beaded structure. We are currently attempting to isolate this entity and determine by usual criteria whether it is a third amebal virus (Mattern, Diamond, Kontomis).

(c) To date we have been unsuccessful in attempts to induce amebal viruses to replicate in vertebrate cells. We are continuing our efforts using chemicals known to facilitate virus uptake (Kontomis, Mattern).

(d) Some strains of amebae produce clinical, or only occasionally detectable, quantities of virus. Some of the HB:3:1 ameba clones behave in this manner. We have been able to appreciably increase virus production by BUDR in some "low producer" strains, and have been able to detect an increase in production in one "high producer" strain (Diamond, Marino, Hruska, Mattern).

(e) To date, one or more morphologically distinct viruses have been isolated from 10 different axenized strains of E. histolytica. Virus host ranges on heterologous strains of amebae have been tested and found different for each virus isolate. All virus isolates have been examined by electron microscopy. All

strains of E. histolytica carry a polyhedral virus. To date only the ABRM and Rahman strains also are capable of inducing an infection with the filamentous virus. In addition, Rahman, 200, and on one occasion amebal strain HM-302 induced production of an intranuclear, beaded structure described above, which is currently under study (Diamond, Mattern, Daniel, Hruska, Kontonis).

2. Potential viruses in other pathogenic protozoa. Initial attempts to isolate lytic agents from Trichomonas vaginalis have been unsuccessful. We are continuing with attempts to induce possible viruses and to employ polymers which are known to facilitate virus uptake by other cell systems.

3. Collaborative studies with investigators of the NCI. (a) We have examined human amnion and lung cells infected with S+L- murine sarcoma virus and ascertained that they probably do not produce C-type particles (Pappageorge et al.).

(b) We have examined murine sarcoma transformed cells in which flat revertants have been induced by fluorodeoxyuridine and colcemid and confirmed production of C-type particles in MuLV rescuable and reverse transcriptase positive clones (Nomura et al.).

(c) We have investigated the tumorigenicity for Balb/C mice of certain spontaneous revertants of Kirsten murine sarcoma virus transformed Balb/3T3 cells. This property was correlated with many (11) other characteristics of these cells (Nomura et al.).

Significance to Biomedical Research and the Program of the Institute

These studies are intended to contribute to our knowledge of the processes by which both RNA and DNA viruses replicate. The ultimate goal, which is finding means of interrupting the viral replication cycle, will undoubtedly require approaches involving multiple disciplines, including such electron microscopic studies.

(1) The possible medical significance of protozoal viruses is being investigated.

(2) The nature of the apparent lysogenic state of the ameba virus is being further investigated.

(3) Studies on the mechanisms of murine sarcoma transformation and reversion of cells may provide clues as to basic processes in human malignancies.

Proposed Course of the Project

Continued intensive study of all phases of the new animal viruses is planned in collaboration with LPD. It is also planned to continue exploration as to the possibility that other human protozoal pathogens may have indigenous viruses. Continued studies on a possible relationship between viruses and animal pathogenicity are in progress.

Publications:

Nomura, S., Fischinger, P. J., Mattern, C. F. T., Gerwin, B., and Dunn, K. J.: Revertants of mouse cells transformed by murine sarcoma virus II. Flat variants induced by fluorodeoxyuridine and colcemid. Virology 56:158-163, 1973.

Hruska, J. F., Mattern, C. F. T., and Diamond, L. S.: Viruses of Entamoeba histolytica IV. Studies on the nucleic acids of filamentous and polyhedral viruses. J. Virol. 13:205-210, 1974.

Mattern, C. F. T., Hruska, J. F., and Diamond, L. S.: Viruses of Entamoeba histolytica V. Ultrastructure of the polyhedral virus V361. J. Virol. 13:247-249, 1974.

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Serial No. NIAID-71-01
1. Laboratory of Viral Diseases
2. Cellular Virology
3. Bethesda, Maryland

PHS-416
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Biological Ultrastructure

Previous Serial Number: SMLE

Principal Investigator: Dr. C. F. T. Mattern

Other Investigators: Wendell A. Daniel, M. S.
Nava Paran, M. S.

Cooperating Units: Dr. Bronislaw M. Honigberg, Dept. Zoology, University of Massachusetts
Dr. Ruth Portel, LPD, NIAID, 122-J

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	12/12		12/12	24/12
Professional	6/12			6/12
Other	6/12		12/12	18/12

Project Description:

Objectives

To study the ultrastructure of protozoa, metazoa, and vertebrates with particular emphasis on the correlation of ultrastructure and function. Organisms and organs under study are selected both to study phylogenetic kinships and certain details of embryogenesis.

Methods Employed

High resolution electron microscopic observations are made in sectioned cells stained by conventional methods and specific histochemical reactions and by autoradiographic electron

microscopy in order to elucidate the chemical composition and function of select organelles.

Major Findings

(1) Continued studies on the ultrastructure of vertebrate and invertebrate contact chemoreceptors are designed to further test a working hypothesis implicating a role of acetylcholine in the primary events of taste. Not only has acetylcholinesterase been localized in the taste pore as well as in previously described areas (Paran and Mattern) but preliminary studies with fluorescent bungarotoxin have demonstrated the presence of an acetylcholine receptor in the pore (Pertel and Paran).

(2) An ultrastructural study of division forms of Trichomonas vaginalis has revealed a number of interesting features. Some details of the division of the basal ont (kinetosomes, pelta, etc.) and an extranuclear microtubular spindle have been determined. Stages in the division of the nucleus and migration of nuclei and blepharoplasts to opposite poles followed by cell division have been elucidated (Daniel, Mattern, Honigberg).

Significance to Biomedical Research and the Program of the Institute

The taste apparatus provides a unique system for the study of sensory function since the taste cells are in contact with the external (oral) environment and can be treated locally as well as readily biopsied. We have provided the first clues as to the mechanism of contact chemoreception by demonstrating the presence of acetylcholinesterase in the taste pore along with the probable localization of an acetylcholine receptor in this area. Further study is needed to evaluate the precise roles of the 3 or possibly 4 types of cell found in the mammalian taste bud.

Study of division forms of T. vaginalis revealing an extranuclear spindle provides additional insights into trichomonad division of possible significance in terms of finding therapeutic adjuncts.

Proposed Course of the Project

These comparative ultrastructural studies of the anatomy of protozoa, metazoa, vertebrate cells and organs will be continued. It is technically feasible to identify cells producing acetylcholine by means of electron microscope histochemical

localization of choline acetyl transferase. Likewise we believe it possible to localize sites of binding of labelled bungarotoxin thus identifying those cells which have acetylcholine receptors. These studies have the potential of providing a highly detailed model of the initial events in the process of taste. Five additional papers are currently being prepared for publication. They represent the culmination of almost 5 years of work and many have become publishable only since some additional understanding of the taste process.

Publications:

Carp, R. R., Mattern, C. F. T., and Monieberg, B. H.: Study of Dientamoeba fragilis I. Electron microscopic observations of the binucleate stages II. Taxonomic position and revision of the genus. J. Protozool. 21:69-82, 1974.

Mattern, C. F. T., and Paran, N.: Evidence of a contractile mechanism in the taste bud of the mouse fungiform papilla. Exp. Neurol, 1974, in press.

Paran, N., and Mattern, C. F. T.: The distribution of acetylcholinesterase in buds of the rat vallate papilla as determined by electron microscope histochemistry. J. Comp. Neur., 1974, in press.

Serial No. NIAID-71-H
1. Laboratory of Viral Diseases
2. Cellular Virology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Host defenses against viral infection: Interferon and Immunity

Previous Serial Number: SAME

Principal Investigator: Dr. Samuel Baron

Other Investigators: Charles E. Buckler

Cooperating Units: Dr. F. Dianzani, University of Turin
Dr. L. Savicki, New York Med. College
Dr. G. Scarnunis, Institute of Microbiology Buenos Aires, Argentina
Dr. Brian Murphy, LID, NIAID
Dr. R. Chanock, LID, NIAID
Dr. P. Price, Microbiological Associates
Dr. E. Zimmerman, Microbiological Associates
Dr. R. Herman, LPD, NIAID
Dr. Michael Worthington, Boston City Hospital

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	36/12	6/12*		42/12
Professional	24/12			24/12
Other	12/12	6/12		18/12

*Stride

Contract Support:

Contract Number	Project Officer	Contractor	Contractor's Project Director	Annual Funding Level
NIH73-C-654CC	Dr. S. Baron	North American Biologicals, Inc.	Dr. R.R. Rafajko	$52,00
AI-21266	Dr. S. Baron	New York Medical College	Dr. M. Galin	$44,275

Project Description:

Objectives

To continue to elucidate mechanisms which control host defenses (interferon and immunity) during viral infections of the whole animal and of cultured cells so that methods might be developed to employ these mechanisms to control human viral infections.

Methods Employed

The interferon system and the immune system were studied in vitro using a variety of cell cultures from different animal species including man. In vivo studies of host defenses during viral infection were conducted in mice and rabbits. The infectious agents utilized in these studies were vaccinia virus, vesicular stomatitis virus, Sindbis virus, Newcastle disease virus, herpes simplex virus, chikungunya virus and mouse leukemia virus. The materials employed included interferons from various species, antibody, both sensitized and normal spleen cells, interferon inducers such as polyinosinic.polycytidylic acid, metabolic inhibitors such as cycloheximide and actinomycin D, and the immunosuppressive agent, cytoxan.

Major Findings

1. Interferon. A study was undertaken to help determine whether the intact virions of a nonreplicating single-stranded RNA virus could stimulate interferon production. Rat embryo cells

were stimulated with the interferon inducing chikungunya virus under conditions where virus replication was completely inhibited. The finding that the stimulated cells produced full yields of interferon under these conditions indicated that a component of the input virion stimulated production of the interferon. Since viral and other nucleic acids are the most general inducers of interferon, and since the input chikungunya virion contains single-stranded RNA, it seems probable that at least certain single-stranded viral RNAs can stimulate interferon production.

Studies with toxoplasma infection in tissue culture were conducted to determine whether interferon or an interferon inducer, poly I.poly C could modify the growth of L. condii in several different types of cell culture. Pretreatment of a variety of human, rabbit, and mouse cells with poly I.poly C induced interferon within 24 hours except for L-cells where no interferon was induced. Inhibition of toxoplasma multiplication occurred in all the treated cell types including the L-cells which did not produce interferon. Further, the inhibition of toxoplasma multiplication did not begin until 72 hours whereas the antiviral state was induced by 24 hours. These findings in contrast to reports in the literature, suggest a non-interferon mechanism for inhibition of toxoplasma by poly I.poly C. Finally, a mouse interferon exerted antiviral effect in mouse L cells but did not inhibit the multiplication of L. Condii. This finding also indicates that interferon may not be inhibitory for toxoplasma multiplication.

Poly I.poly C, an interferon inducer with potential for application to man, was found to enhance in vitro transformation to malignancy of rat embryo cells. Low passage Fisher rat embryo cells were transformed when poly I.poly C was added to cells newly infected with murine leukemia virus. Poly I.poly C had no transforming effect when added to virus free cultures or to the low passage cells chronically infected with leukemia virus. High passage Fisher rat embryo cells were transformed by poly I.poly C in either the absence or presence of leukemia virus. The findings indicated that further studies are needed on the possible oncogenic potential of poly I.poly C.

Interferon produced by cells of a given genus generally exert antiviral activity best in cells of the same genus, partially in cells in closely related genera and rarely in distantly related genera. During a study of cell typing by

interferons, it was observed that monkey interferon unexpectedly protected rat cells against virus infection. Several different types of monkey interferon manifested this heterologous activity. The heterologous activity also manifested many of the properties of interferon in that (1) it was stable at pH 2, (2) it was inactivated by trypsin, (3) it was cell-bound, (4) it was inhibited by actinomycin D but not by cycloheximide, (5) it was active against unrelated viruses and (6) it was inactive on other heterologous cells. In this way it was established that monkey interferon is one of the exceptional interferons which act heterologously in rat cells.

A study was undertaken of the effect of viral infection on subsequent infection by the avian malarial parasite P. gallinaceum in chick embryos. The results of interference studies between several viruses and the malarial parasite suggested that those viruses which are able to induce interference (perhaps mediated by interferon) were also able to inhibit parasitic growth.

2. Immune mechanisms. Immunosuppression studies were carried out in the mouse utilizing antithymocyte serum, X-irradiation and neonatal thymectomy as the immunosuppressive agents. Antithymocyte serum and X-irradiation potentiated primary systemic infection with vaccinia virus. Formation of neutralizing antibody was suppressed and viremia was prolonged. The potentiation of the virus infection was largely reversed by passive transfer of physiologic amounts of antibody at the appropriate time. Neonatally thymectomized mice, on the other hand, formed neutralizing antibody normally and recovered normally from primary vaccinia virus infection although their level of cell mediated immunity was drastically reduced. Levels of interferon in the serum were not influenced by these methods of immunosuppression. The results suggest a critical role for antibody but not for cellular immunity in recovery of mice from primary systemic infection with vaccinia virus. The effects of interferon induction and passive antibody on the course of fatal systemic vaccinia virus infection in immunosuppressed mice were studied. The combination of an interferon inducer plus antibody was considerably more effective than therapy with either of these agents alone when administered late in the course of this systemic infection. These findings suggest that combined therapy with an inducer of interferon and specific antibody to virus might be effective in immunologically incompetent hosts with certain severe viral infections.

A study of experimental herpes simplex virus keratoconjunctivitis of the rabbit was undertaken to compare virus production, interferon and antibody production in those eye tissues which show typical clinical signs. Virus titers were not always correlated with onset of clinical signs. Similarly, appearance of local antibody did not correlate with elimination of herpes simplex virus from the cornea or conjunctiva. Stromal disease coincided with the appearance of serum antibody and iritis ensued two days earlier than local antibody in this tissue. Since iris antibody exceeded serum antibody by a factor of 10 on day 5 it is likely that it was produced by immunocompetent cells invading the iris. The inability of the herpes simplex virus infection to induce detectable concentrations of interferon and the relative insensitivity of herpes simplex virus to the action of interferon indicates that the interferon system may not play an important role during natural recovery from this infection of rabbit eyes.

Significance to Biomedical Research and the Program of the Institute

The finding that a non-replicating, single-stranded RNA virus can stimulate interferon production indicates that single-stranded viral RNA and perhaps other single-stranded RNAs are capable of inducing good yields of interferon. Thus, the search for new inducers of interferon can include single-stranded RNAs in addition to the double-stranded RNAs being examined currently. The finding that interferon itself exerted no inhibitory action on the toxoplasma organism helps correct some misconceptions in the literature. The finding that poly I.poly C may act as a transforming agent in an extremely sensitive system indicates a possible undesirable side effect of polynucleotides which are being considered for use as interferon inducers in man. The study of viral interference with the growth of the avian malarial parasite *P. gallinaceum* provides additional supporting evidence for the concept that interferon and interference may affect the growth of the malarial organisms. Studies of the disseminated vaccinia virus infection in immunosuppressed animals which mimics certain disease states in man, indicates that antibody but not cellular immunity plays an essential role as a host defense in the primary viral infection. The demonstration that an interferon inducer plus antibody provided synergistic therapeutic action in this infection suggests potential application to similar viral infections of man. The

immunopathogenic study of herpes simplex virus keratoconjunctivitis of the rabbit indicated that cell mediated immunity might be a more important host defense factor than is antibody or interferon.

Proposed Course of the Project

The studies of induction of interferon by single-stranded RNAs will be continued as a search for additional polynucleotides which may serve as effective and practical inducers of interferon to control human disease. Collaborative clinical studies using interferon and interferon inducers will be continued as promising areas for study are developed. Studies designed to elucidate the immunopathological development of viral disease using the immunosuppressed animal and reconstitution experiments will be continued. The vaccinia virus phase of this study is largely complete and a shift in emphasis towards the herpes viruses will be made since it is likely that the immunologic and interferon mechanisms are quite different for the two virus infections.

Honors and Awards (Dr. Baron):

Associate Editor, American Journal of Epidemiology

Associate Editor, International Journal of Cancer

Member of Committees for American Association of Immunologists, and American Society for Microbiology

Member of the Board of Directors of the American Type Culture Collection

Member of the U. S. Army Contract Review Group on virus infections.

Invited lectures at Universities, Research Institutes, and Symposia

Publications:

Baron, S., Finter, N. B., Galasso, G. J., Glasgow, L. A., Levy, H. B., and Youngner, J. S.: Interferon. Science 158: 776-784, 1967.

Baron, S., Cathala, F., and Levy, J. P.: Le problème de l'interferon. Therapeutique 45: 511-532, 1971.

Price, P. J., Sun, L. A., Zimmerman, E. M., Spahr, C. J., Gazdar, A. F., and Baron, S.: Enhancement of in vitro transformation by polyinosinic-polycytidylic acid. J. Natl. Cancer Inst. 50: 1293-1300, 1973.

Schaumis, G., Weissenbacher, R., Chouchavech, E., Savichi, L., Galin, M. A., and Baron, S.: Growth of Toxoplasma gondii in various tissue cultures treated with In.Cn or interferon. Proc. Soc. Exp. Biol. Med. 143: 1153-1157, 1973.

Uhlendorf, C. P., Zimmerman, E. M., and Baron, S.: Heterologous activity of monkey interferons. Proc. Soc. Exp. Biol. Med. 144: 626-632, 1973.

duBuy, H., Baron, S., Uhlendorf, C., and Johnson, M. L.: Role of interferon in murine lactic dehydrogenase virus infection, in vivo and in vitro. Infect. & Immun. 8: 977-980, 1973.

Herman, R., Shiroishi, T., and Buckler, C. E.: Viral interference with exoerythrocytic forms of malaria (Plasmodium gallinaceum) in ovo. J. Infect. Dis. 126: 146-155, 1973.

Worthington, M.: Mechanism of recovery from systemic infection with vaccinia virus. II. Effects of X-irradiation and neonatal thymectomy. J. Infect. Dis. 127: 512-517, 1973.

Worthington, M.: Mechanism of recovery from systemic infection with vaccinia virus. III. Effect of antithymocyte serum. J. Infect. Dis. 127: 518-524, 1973.

Worthington, M., and Baron, S.: Role of non-specific and immune factors during viral infections. In Braun, W. and Uhlar, J. (Eds.): Non-Specific Factors Influencing Host Resistance. Karger, Basel, pp. 412-423, 1973.

Worthington, M., and Baron, S.: Effect of polyriboinosinic-polyribocytidylic acid and antibody on infection of immunosuppressed mice with vaccinia virus. J. Infect. Dis. 126: 308-311, 1973.

Savicki, L., Chovchavech, E., Weissenbacher, M., Baron, S., and Galin, M.: Pathogenetic studies of herpes simplex virus infection of the rabbit eye. Proc. Soc. Exp. Biol. Med. 144: 703-708, 1973.

Murphy, B. R., Baron, S., Chalhub, E. G., Uhlendorf, C. P., and Chanock, R. M.: Temperature-sensitive mutants of influenza virus. IV. Induction of interferon in the nasopharynx by wild-type and a temperature-sensitive recombinant virus. J. Infect. Dis. 128: 480-483, 1973.

Dianzani, F., Publiese, A., and Baron, S.: Induction of interferon by nonreplicating single-stranded RNA virus. Proc. Soc. Exp. Biol. Med. 145: 426-433, 1974.

Serial No. NIAID-71-I
1. Laboratory of Viral Diseases
2. Molecular Virology
3. Bethesda, Maryland

PHS-431
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The interaction between latent and slow viruses, especially the Lactic Dehydrogenase Virus (LDV) and the subcellular elements of the host cell (mouse macrophages for LDV). Use of the LDV-modified macrophages model for the study of role of macrophages in the defense mechanisms of the host.

Previous Serial Number: SAMP

Principal Investigator: Dr. Herman G. DuBuy

Other Investigators: None

Cooperating Units: Dr. C. F. T. Mattern, LVD, NIAID, 71-G
Dr. Samuel Baron, LVD, NIAID, 71-H

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total				
Professional				
Other				

Project Description:

Objectives:

Study of the factors, both in vivo and in vitro, that determine whether a virus kills the host, becomes chronic, or is eliminated, and of factors which modify the outcome of the virus infection: interferon and its inducers, humoral antibody, cellular defense factors, especially those of macrophages, the target cells of LDV.

Methods Employed

The murine Lactic Dehydrogenase virus is used as a model for other chronic viral diseases and latent infections because it is an RNA membrane virus which multiplies exclusively in mouse macrophages, leading to a chronic viremia without overt morbidity of the host. Subprojects have been developed which allow the use of this model for the determination of in vivo and in vitro changes of macrophages functions (induction of interferon by viruses multiplying in macrophages, action of exogenous interferon or its inducers on the multiplication of these viruses, clearance of i.v. introduced viruses, or interferon, or serum enzymes, etc.).

Major Findings

The study on the role of interferon (IF) in murine LDV infection, in vivo and in vitro showed that LDV is a poor IF inducer, and that the virus has a relatively low sensitivity to IF: in macrophage cultures it was 30% less sensitive than Vesicular stomatitis virus, and 5% less than Sendai virus. Prolonged treatment with IF still did not lead to a cure: in part because a slow toxicity of the IF killed the macrophages after a few weeks of treatment.

Homologous interference was found, when macrophage cultures of chronically LDV infected mice and of control mice were reinfected with LDV. After in vitro superinfection with LDV the superinfected macrophages showed a reduced LDV titer when compared with macrophages from control mice, but an increased titer when compared with the titer of nonsuperinfected macrophages from chronically LDV-infected mice.

The results on the effect of IF inducer In.Cn on the LDV titers in vivo and in vitro are incorporated into a manuscript to be presented for publication.

The study of agents which change macrophage functions, and might lead to elimination of LDV infection of macrophages was continued by using i.p. injection of silica, which has been reported to kill macrophages. Contrary to expectation it was found that the LDV titers were increased. Preliminary results indicate that damage to the macrophages induced proliferation of new macrophages, thus producing higher LDV titers.

Significance to Biomedical Research and the Program of the Institute

The study of the chronic viremia caused by LDV contributes to the understanding of other chronic or latent viral infections. Because of the specific involvement of macrophages in the macrophage-LDV model, it may further clarify the role of macrophages in resistance to chronic viral diseases.

Proposed Course of the Project

Continuation of the study to eliminate chronic LDV viremia by using less toxic IF preparations and MIF-IF preparations when they become available.

Continuation of the studies on the effect of silica on macrophages.

Publications:

duBuy, H., Baron, S., Uhlendorf, C. and Johnson, K.: Role of interferon in murine lactic dehydrogenase virus infection, in vivo and in vitro. Infect. & Immun., 8:977-984, 1973.

Serial No. NIAID-71-J
1. Laboratory of Viral Diseases
2. Cellular Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: "Slow" and Oncogenic Viruses

Previous Serial Number: SAHE

Principal Investigator: Dr. Kenneth K. Takemoto

Other Investigator: Dr. Michael F. Mullarkey, LVD, NIAID
Dr. M. A. Martin, LBV, NIAID
Dr. G. Khoury, LBV, NIAID
Dr. C. A. Garon, LBV, NIAID
Dr. P. Howley, LBV, NIAID

Cooperating Units: Dr. A. S. Rabson, Laboratory of Pathology, NCI
Dr. R. M. Blaese, Laboratory of Immunophysiology, NCI

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	36/12			36/12
Professional	24/12			24/12
Other	12/12			12/12

Project Description:

Objectives

(1) To characterize biologically and biochemically viruses which are responsible for slow, chronic, and persistent infections and to study their relationship to oncogenic viruses. (2) To study the virus-cell interactions of these viruses in their permissive and nonpermissive hosts. (3) To evaluate their role in human disease.

Methods Employed

Standard cell culture and virologic as well as biochemical methods are used.

Major Findings

The past year's efforts have been directed toward characterizing as completely as possible, the newly discovered human papovaviruses. Our results as well as those from several other laboratories indicate that there are at least 2 distinct papovaviruses of the polyoma SV40 subgroup which are human pathogens. The BK and JC viruses are the prototype strains for based on serologic studies, they are common viruses which infect 80% of humans. The JC virus is probably the virus responsible for the demyelinating disease, progressive multifocal leukoencephalopathy (PML). It has been isolated from 1[illegible] cases of PML thus far.

1. Transformation studies: The oncogenic properties of BK virus were investigated by, in vitro as well as in vivo studies. No tumors have developed in newborn hamsters inoculated intracerebrally, intraperitoneally, or subcutaneously. Newborn mice inoculated with BKV also have shown no signs of developing disease. Through the National Cancer Institute, 5 newborn rhesus monkeys were inoculated with purified BKV by various routes. Two developed lymphadenopathy 1 month after virus inoculation. All responded with high antiviral antibody and these animals will be observed for 2 or 3 years for development of disease.

In cell cultures, there has been no evidence for viral transformation induced by BKV in rat, mouse, or hamster cells. However, human fetal brain cell cultures have been transformed by BKV. Transformed lines were obtained after lytic infection which was followed by recovery of the culture. The transformed cells are unusual in that the virus persists indefinitely despite rigorous cloning procedures and antibody treatment. Another unusual feature of these transformed cells is that the tumor antigen (T-antigen) is not synthesized except in cells undergoing productive infection (about 5-10% of the cells).

2. Biochemical and biophysical characterization of BKV. [illegible] have reported that BKV and SV40 share common structural as well as virus-induced T-antigens. Analysis of the structural polypeptides of BKV showed that there are [illegible] proteins in the virion. The major protein (VP-1) is slightly smaller than that of SV40. Antigenic analysis by immunodiffusion technique failed

to reveal common antigens between BKV and SV40. However, by sensitive FA techniques, cross-reactions between BK and SV40 were noted, indicating that 2 viruses are related but distinct.

The extent of relatedness between BKV and SV40 was further analyzed by DNA hybridization techniques (these studies were done in collaboration with Drs. Martin, Howley, and Khoury of the Laboratory of Biology of Viruses). Preliminary evidence indicates that the 2 viruses may share common sequences to the extent of 15 to 25 percent.

3. The JC virus. Efforts to study the JC virus have been hampered by a lack of a good host cell to grow the virus. Thus far, it appears to grow only in spongioblast cells derived from human fetal brain cultures. Its replication in these cells is slow, requiring up to 1 month before cellular destruction occurs.

4. Isolation of papovavirus from brain tumor and urine of Wiskott-Aldrich patient. A virus antigenically similar to BKV was isolated from a brain tumor (reticulum cell sarcoma) and also the urine of a patient with Wiskott-Aldrich Syndrome, a sex-linked immunodeficiency disease in which there is a high incidence of malignancy. Although a direct relationship between virus and tumor could not be established in these studies, this is an important lead which is being further pursued. In another patient with Wiskott-Aldrich Syndrome, who had high antibody titers to BKV, papovavirus was also isolated from his urine. This virus is currently being characterized.

Significance to Biomedical Research and the Program of the Institute

The knowledge that DNA viruses of the papova group which have long been known for their oncogenic potential are widespread in the human population opens up a new area of investigation concerning the importance of these viruses not only in oncogenesis, but in other heretofore unexplained diseases of man. Serologic evidence indicates that children are infected early in life, and it is likely that the virus may persist indefinitely. At least one of the newly isolated viruses (JC) causes a slow, demyelinating disease, progressive multifocal leukoencephalopathy.

Proposed Course of the Project

1. Efforts will be continued to completely characterize the prototype human papovaviruses, JC and BK. These include biologic

as well as biochemical studies which are being carried out in collaboration with several other groups.

2. The significance of these viruses in relation to human disease will be assessed. In particular, studies on isolation of these viruses from immune deficiency diseases as well as from patients with neoplastic disease will be continued.

Honors and Awards:

1. Current Information Concerning Human Papovaviruses, presented at the Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

2. Human papovaviruses and their Possible Role in Human Cancer. Eighth Annual Meeting of the Virus Cancer Program, Hershey, Pa.

Publications:

Takemoto, K. K., and Mullarkey, M. F.: Human papovavirus, BK strain: Biological studies including antigenic relationship to simian virus 40. J. Virol. 12:625-631, 1973.

Takemoto, K. K., and Mullarkey, M. F.: Human papovaviruses. In Biohazards in Biological Research, Cold Spring Harbor Laboratory, 133-138, 1973.

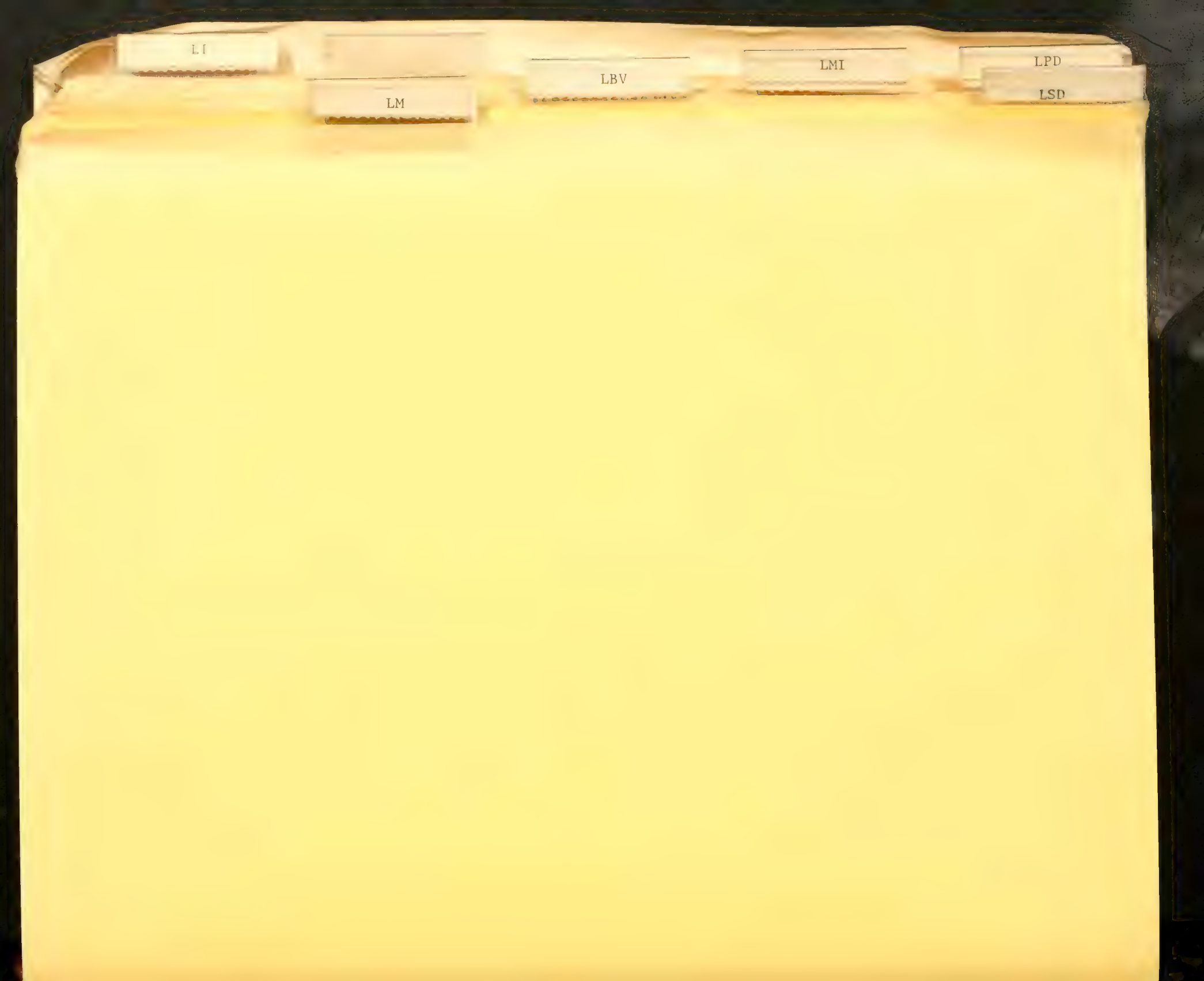
Khoury, G., Byrne, J. C., Takemoto, K. K., and Martin, M. A.: Patterns of simian virus 40 DNA transcription. II. In transformed cells. J. Virol. 11:54-60, 1973.

Law, L. W., and Takemoto, K. K.: Specific transplantation antigens of murine neoplasms induced by visna and progressive pneumonia viruses. J. Natl. Cancer Inst. 50:1675-1678, 1973.

Martin, M. A., Gelb, L., Takemoto, K. K., Nathans, D., and Lee, T.: Characterization of "heavy" and "light" SV40-like particles from a patient with PML. Virology, 1974, in press.

Mullarkey, M. F., Bruska, J. F., and Takemoto, K. K.: Comparison of two human papovaviruses with SV40 by structural protein and antigenic analysis. J. Virol., 1974, in press.

Takemoto, K. K., Ralson, A. S., Mullarkey, M. F., Blaese, R. M., and Garon, C. F.: Isolation of papovavirus from brain tumor and urine of a patient with Wiskott-Aldrich syndrome. Science, 1974, in press.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LABORATORY OF MICROBIOLOGY

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PHS-NIH
Summary Statement
Laboratory of Microbiology, NIAID
July 1, 1973 through June 30, 1974

Introduction

Beginning in June 1973, administrative action was initiated to disestablish the Laboratory of Microbiology, as a result of strictures placed on NIAID by NIH and Departmental levels to lower the personnel ceiling by June 30, 1974. On June 24, 1973, the Mycoplasma Section was transferred to the Laboratory of Infectious Diseases, and a part of the Medical Mycology Section was transferred to the Laboratory of Clinical Investigation, thereby removing 7 personnel from LM. During the year, eight technical personnel and one clerk-typist accepted positions to fill vacancies elsewhere, as did one Commissioned Officer (Scientist). One technician retired. Seven investigators and 3 technicians--for whom no relocation has been achieved--remain as of the date (May 9, 1974) of this writing. Despite the loss of morale and the continuing loss of technical assistance, the remaining investigators have completed or continued work previously listed in 10 projects, which are so listed here again under the original NIAID project numbers.

It is anticipated that one investigator and one technician will be reassigned to the Laboratory of Streptococcal Diseases; that one investigator (CO) will be reassigned to the Rocky Mountain Laboratory; and that one one investigator (CO) will retire sometime after the start of FY 75. Although present projects may terminate for these reasons, there is still uncertainty as to timing, and to administrative responsibility and ultimate location of the other personnel. Therefore, no individual project report contains notice of termination at this time.

Other personnel leaving LM, as it expires as of the new fiscal year, will be Dr. Sang Jae Kim, Visiting Fellow from Korea; and Dr. Errol Reiss, Post-Doctoral Fellow from Rutgers.

The principal mission of the Laboratory of Microbiology has been construed to be the advancement of basic knowledge of prokaryotic microorganisms. Organisms studied included bacteria, fungi, mycoplasma, and bacterial viruses. Areas of research included genetics, metabolism, serology and immunology, taxonomy, ultrastructure, biochemistry, and all interrelations thereof. Particular emphasis was given to structure-function relations of such isolatable microorganismal components as cell wall, cell membrane, organelles such as mesosomes, the DNA genetic apparatus, and chemical entities such as structural proteins, enzymes, and other biologically active molecules. Most of these latter functions will be incorporated in the new Laboratory of Streptococcal Diseases.

LI
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CO_2 (bicarbonate) Imposes Absolute Control Over Cell Growth

A broad spectrum of bacteria representative of diverse nutritional and morphological types were shown to require CO_2 or bicarbonate for growth. The two strict aerobes tested responded specifically to bicarbonate concentration, whereas the others were CO_2-sensitive. Initiation of growth was CO_2/bicarbonate dependent and the usual lag period was eliminated when the correct concentration of CO_2/bicarbonate was provided. Physiologically old inocula grew at the same rapid experimental rate as young inocula, although they often had a quantitatively different initial requirement for CO_2/bicarbonate. The data shows that the lag period is not a physiological state of cells; it is a condition imposed by a limiting nutrient. Cultures continued to require CO_2/bicarbonate throughout growth and removal caused an abrupt cessation of growth. Other experiments showed that CO_2 was not exerting its effect by providing C4 dicarboxylic acids. CO_2/bicarbonate appears to have another growth controlling function which has never been described. The recognition of this new control system has important implications as a target for various biological and chemical agents which affect cell growth. (Repaske)

Possibilities for Mesosome Function Clarified

Many speculations have been made on the role of the mesosome, a septally-located invagination of cytoplasmic membrane which is considered a bacterial organelle. No good evidence for any role has been presented. An essential first step successfully utilized in this laboratory (using Staphylococcus aureus) has been to isolate mesosomal vesicles in order to be able to compare their composition and, ultimately functions, with those of similarly isolated membrane. Earlier results showed minor quantitative differences in protein and lipid but improved methods now demonstrate that high mesosomal levels of glycerol phosphate reflect the presence of cell lipoteichoic acid (LTA) exclusively in mesosomal vesicles. Essentially none was found in membrane, despite the convention in the literature of referring to this compound as "membrane" teichoic acid. A similar disparate distribution was found in 6 other strains of gram-positive bacteria, representing the genera Staphylococcus, Streptococcus, Bacillus, Micrococcus, and Lactobacillus. With this unequivocal mesosome marker, attention can now be directed to biosynthetic activities and other roles in which the magnesium-sequestering capability of LTA may be important. Other studies this year show that specific lipids (phosphatidyl glycerol, cardiolipin, monoglucosyl diglyceride, diglucosyl diglyceride, phosphatidyl glucose, carotenoids, and vitamin K_2), although not qualitatively different, are all present in greater amounts per unit weight in mesosomal vesicles than in cytoplasmic membrane--suggesting a role in synthesis of new membrane. Previous ideas of mesosomes as major sites of respiratory activities seem ruled out by this year's culmination of studies showing that cytochromes, oxidases, and dehydrogenase activities

for four different substrates, are all associated primarily with the cytoplasmic membrane fraction. Furthermore, degradative enzymes (alkaline and acid phosphatases, 5'-nucleotidase, adenosine triphosphatase, nuclease) were found primarily in periplasm or extracellular fluid, and not in either membrane nor mesosomal vesicles: only insignificant by greater levels of protease activity were associated with the latter. Staphylococcal alpha toxin was also found to be chiefly periplasmic. These results negate another postulated function of mesosomes as lysosome-like bodies which store degradative enzymes. Only penicillinase, after induction in a non-constitutive strain of S. aureus 80/81, was most concentrated in mesosomal vesicles--a finding consistent with results of other investigators in Bacillus. All in all, the accumulating evidence suggests that one of the earliest concepts of mesosome function, which arose purely from its ultrastructural origin from membranous septum at the developing new cross-wall, may be correct; namely, that it is involved in membrane and cell wall biosynthesis. Additionally, it is logical that protection (in the form of penicillinase) against an antibiotic effective only at the major site of new wall synthesis, would also be concentrated at this same cross-wall location. (Theodore, Huff, Beining, Prescott, and Cole)

New Method for Isolation of Streptococcal M Protein

Group A streptococcus, M-type 4 (CDC strain 0107) has been studied as a representative of "difficult" but common serotypes whose type-specific and protection-inducing M proteins would need to be included in any useful polyvalent immunizing preparation. M proteins from these types have been little studied, but are known to offer problems in purification, in yields, and in co-purification of a lipoproteinase or serum opacity factor (SOF). Last year's efforts highlighted M yields and purification from culture supernatant fluids. This year's studies concentrated on extraction (and subsequent purification) of M directly from cells, by new methods. Treatment at pH 4.4 with molar lithium chloride (LiCl) released material reactive with specific (CDC) type 4 antiserum. Varied treatments (ammonium sulfate precipitation, column chromatography, etc.) of this material revealed at least 5 antiserum-reactive components, of which at least 2 were dialyzable: one of these was of basic character, as shown by acid gel electrophoresis. In alkaline gels, more protein-staining components that appear to be antiserum-precipitable, were found. Only equivocal tests of SOF activity were found in LiCl fractions, although culture supernates were strongly positive. The results suggest that SOF may be separable from antiserum-reactive components, but whether these latter all represent only M or what the non-reactive protein components may be, remains to be determined. (Schade)

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Plant, Animal, and Microbial Glycolipids Related

Extraction of certain plant substances (from carrot, parsnip, and spinach) with chloroform-methanol, followed by DEAE-cellulose and thin-layer chromatography of the extracted lipids, yields a pure glycolipid hapten which exhibits a high level of reactivity with complement-fixing (CF) antibodies to Mycoplasma pneumoniae. Chemical characterization of the carrot hapten indicates that the glycolipid contains glycerol, fatty acid esters, and carbohydrate (galactose) in a molar ratio of 1:2:2, which is similar in structure to digalactosyl diglycerides from other plants. A similar galactose-containing glycolipid also reactive with M. pneumoniae antisera, was isolated from human brain: galactose was shown to be the antigenic determinant, presumably identical in its stereospecificity to the galactose known in M. pneumoniae glycolipid. The possibilities of antibodies, directed against plant or microbial glycolipids, which may then be cytotoxic for human nervous or other tissues, raise interesting questions which remain to be explored. (Prescott, Caldes; Channock, Greenberg, Helms, and James, LID)

New Class (Phosphoglycolipids) of Serologically Active Glycolipids Found

Prior studies showed one major and two minor antigenic glycolipids in Groups A and D streptococci. By a new solvent procedure (chloroform-methanol-acetic acid-water), followed by non-lipid removal with potassium chloride, DEAE-cellulose column chromatography, thin-layer silicic acid chromatography, and subsequent two-dimensional chromatography on silica gel plates, one of the minor components was isolated. It has been identified as glyceryl phosphoryl diglucosyl diglyceride. It, as well as the major diglycosyldiglyceride previously found, is serologically cross-reactive by complement fixation with antisera against Groups A and D streptococci and Acholeplasma laidlawii. This finding adds to the variety and complexity of glycolipids which occur in diverse organisms in nature and which appear to be serologically identical. The demonstrated possibilities of "tailoring" antigens by glycolipid-protein reaggregations, is being further explores. (Prescott)

Serologic Relationships and Antigens of Yeasts and Fungi

A close antigenic relationship exists between Saccharomyces telluris serotype A and Candida albicans antigenic Group A. This can be shown by agglutination of whole yeast cells and by immunodiffusion tests of mannans extracted from yeast cells. Although by agglutination of S. telluris A shares an antigenic component with the other two serotypes of this species, and C. albicans A shares antigenic properties with C. albicans B and S. cerevisiae not found in any S. telluris serotype, these respective antigens appear to be quantitatively minor.

A serologically reactive galactomannan (galactose/mannose 2:5) has been isolated from Histoplasma capsulatum. This polysaccharide gives a single precipitin band by immunodiffusion, does not elicit delayed hypersensitivity in sensitized guinea pigs, but does specifically inhibit macrophage migration. A glycoportein extracted from this fungus shows two precipitin bands in immunodiffusion: one, identical with the galactomannan, induces a delayed hypersensitivity reaction, and specifically inhibits macrophage migration.

A cytoplasmic protein, obtained from mechanically disrupted C. albicans yeast cells, has been shown to be reactive by immunoelectrophoresis and in immunodiffusion with sera from infected rabbits and patients with chronic candidiasis. This protein elicits delayed hypersensitivity in sensitized animals and contains no detectable mannose. (Hasenclever, Reiss, NIH; Ellsworth, G.W.U.)

Factors Affecting the Existence of Pathogenic Fungi in Nature

Studies at two different sites colonized by Histoplasma capsulatum indicated that approximately five years after the source of fecal contamination (bird or bat roosts) was removed, soil samples collected randomly varied from 4-8% positive. This low but detectable colonization by the fungus continued for another five years during the course of the study. H. capsulatum was known to be present in the site associated with a bird roost for 11 years and at a site associated with a bat roost for 15 years.

Although many sites where H. capsulatum is known to colonize soil are near or under chiropteran habitations, the addition of bat guano to a measured area with low but detectable amounts of the fungus present did not result in a quantitative change. This lack of response was noted over a three year period. The addition of a soil culture to the area treated with guano failed to establish heavy colonization by the fungus.

Candida albicans was found in one-third of the samples of pigeon droppings collected in urban and rural areas. Earlier studies had shown that the upper and lower gastrointestional tracts of over 50% of feral pigeons cultured were positive for C. albicans. The importance of this source of the yeast as a contagion for humans remains unknown but probably represents a public health hazard. (Hasenclever)

Cellular Immunity in Inbred Guinea Pigs; Animal Models for Infectious and Autoimmune Diseases

Using histocompatible inbred Strain 13 guinea pigs, systemically immunized with living BCG, protection against virulent M. tuberculosis can be adoptively transferred by living lymphoid cells to recipients which do not develop demonstrable delayed hypersensitivity. The imbred guinea pigs also provide an animal

model for multiple sclerosis, demonstrating an age-dependent chronic autoimmune encephalomyelitis: ultrastructural examination reveals repeated episodes of demyelination and remyelination. (Stone)

Serial No. NIAID 61
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The effect of CO_2 on growth of microorganisms

Previous Serial Number: Same

Principal Investigator: Dr. Roy Repaske

Other Investigators: Mrs. Anne C. Repaske, Guest Worker

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	2/12	-	6/12	8/12
Professional	2/12	-	6/12	8/12
Other	-	-	-	-

Project Description:

Objectives:

To confirm the requirement for carbon dioxide or bicarbonate for elimination of the lag period and for growth by using bacteria representative of major nutritional groups.

Methods Employed:

Inocula were grown in defined medium until they reached the desired physiological age. A small inoculum of washed cells was used in a 1 liter fermentor containing the same medium. Cells in the fermentor were grown under a controlled gas environment provided by independently regulated gas flow of N_2, O_2 and CO_2 which permitted rapid and accurate changes in the gas atmosphere as necessary. Growth was monitored and recorded continuously by a spectrophotometer through which a portion of the culture was constantly cycled. The CO_2 versus bicarbonate requirement was determined by employing the Henderson-Hasselbalch relationship.

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Major Findings:

We have been exploiting a very significant earlier observation that the "normal" lag period in growing cultures could be prevented by the simple expedient of altering the CO_2/bicarbonate concentration in the growth medium. The unusual aspect was that aged cells required a different initial concentration of CO_2/bicarbonate. Thus Hydrogenomonas eutropha an aerobic autotroph,was sensitive to bicarbonate ion concentration; physiologically young inocula needed 6.6×10^{-4}M bicarbonate; older cells needed 1.6×10^{-3}M bicarbonate, and stationary phase cells needed 5.3×10^{-3}M bicarbonate. After 1-2 generations, the bicarbonate ion requirement for each type of cell reverted to 6.6x10-4M. The general significance of these observations became apparent when we domonstrated that Streptococcus sanguis, a nutritionally fastidious anaerobic heterotroph, could also be grown without the usual four-hour lag period. This organism, however, responded to the CO_2 concentration and not to bicarbonate; but again the CO_2 required was a function of the physiological age of the inoculum. An unexpectedly important role for CO_2 requirement persisted even after culture growth was well established. This requirement was unambiguous, since growth stopped immediately when CO_2 was deleted from the atmosphere.

Our sampling of organisms was extended to include the facultative anaerobe, E. coli, and the obligate aerobe, Bacillus megaterium both of which were grown in a simple organic medium. Whether E. coli was grown aerobically or anaerobically, an obligatory CO_2 requirement was demonstrated to initiate growth, to grow without a lag period and to sustain growth. B. megaterium was shown to require bicarbonate as did the other strict aerobe, H. eutropha.

Although carbon dioxide fixation by heterotrophic organisms is well known, the CO_2 is fixed primarily into oxalacetate and eventually into aspartic acid. Because (1) aspartic acid supplements to the medium do not spare the CO_2 requirement, even when cells are permeable to aspartate; and (2) acetate grown E. coli continues to require CO_2, another role for CO_2 is suggested for the control of cell growth.

The following statements summarize our data and conclusions:

A. A variety of nutritionally diverse organisms have been tested and without exception all require CO_2 or bicarbonate for growth.

B. The lag period is not a "normal" physiological stage of growth. It represents the culture's response time to produce endogenously a sufficient CO_2/bicarbonate concentration. No lag period is observed when the proper CO_2/bicarbonate concentration is provided. The optimum concentration is quantiatively different for each organism.

C. Both strict aerobic organisms tested were sensitive to bicarbonate ion concentration while the anaerobe (S. sanguis) was sensitive only to CO_2 concentration. Technical limitations of the equipment did not allow us to discriminate between a CO_2 or bicarbonate requirement with the facultative anaerobe (E. coli).

D. Growing cultures continue to require CO_2/bicarbonate; growth stops when CO_2 is deleted. Limiting CO_2/bicarbonate supports culture growth at a limiting growth rate.

E. Carbon dioxide and bicarbonate is some way exert overriding control over growth in an otherwise nutritionally complete medium.

Significance to Biomedical Research and the Program of the Institute:

The cell, the fundamental living unit, is the ultimate focal point of biological and biomedical research. Various aspects of organization and regulation still remain to be elucidated before integrated reactions characteristic of normal whole cells can be understood. An assessment of the cell's response to its chemical and physical environment--its physiology--continues to be the basis for investigating and interpreting normal and abnormal cellular reactions. Cell physiology, therefore, supplies direction as well as meaning to biochemical studies.

Our recent studies may have dramatic implications for growth, a fundamental aspect of cell physiology. Growth of bacterial cells as well as mammalian cells is graphically described by a sigmoid growth curve in which the early period of "no growth" or slow growth is called the lag phase. This descriptive curve found in all texts is firmly established as the "gospel" of the physiology of growth. By demonstrating with a variety of bacteria that the lag phase can be circumvented we have shown that a basic tenet of cell physiology is in error. The lag period is artifically imposed on cultures by a nutritional deficiency of carbon dioxide or bicarbonate. This critical nutrient, carbon dioxide, is so ubiquitous that scientists have neglected to evaluate it quantitatively.

If our findings on the broad sample of bacteria is truly representative for all living cells, a universal preemptive role for CO_2 for growth is suggested. The implications for control of cell growth and interpretation of growth related functions (virus infection, antibiotic inhibition, etc.) are of the greatest importance because this is a parameter of growth that has never been evaluated in terms of a target for deleterious agents or conditions.

Quantitatively different CO_2/bicarbonate requirements when older cells are used as inocula suggests an experimental approach to an aspect of cell aging. It is particularly encouraging that these old cells have the same capacity for rapid growth as younger cells when CO_2/bicarbonate is provided.

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Proposed Course of Project:

The fact that a variety of nutritionally diverse bacteria respond similarly to CO_2/bicarbonate strongly suggests a universal function of CO_2/bicarbonate for all cells. Comparative physiology and biochemistry principles lend sufficient credence to this possibility that these preliminary studies justify a serious effort to identify the enzymatic reaction(s) controlled by CO_2. First, however, it would be of practical as well as theoretical value to extend the general physiological studies to normal and to transformed tissue culture clones.

Biochemical studies will be directed to identify the primary system responding to CO_2/bicarbonate. Representative systems to be tested include: transcription (DNA and RNA), translation, intermediary catabolic and anaplerotic systems, aerobic metabolic systems, energy transducing systems, etc. Once the general system dependent upon CO_2 has been identified, a detailed study of the components of the system will be made to localize the CO_2 sensitive reaction.

Rapid identification of the system(s) which CO_2 influences will be made through active collaboration with investigators who are presently using these systems. The test organisms will necessarily vary and will be determined by "most suitable for evaluation".

Honors and Awards:

1. Served as Program Director for Biochemistry for the National Science Foundation from September 1973 through June 1974.

2. Invited Participant in Bioenergy Conversion Workshop, Wash. D.C. Sept. 3-5, 1973.

3. Invited Seminar. National Science Foundation., Wash. D.C. May 22, 1974. "Carbon Dioxide Requirements for Cell Growth."

4. Invited Participant in International Symposium on N_2-Fixation. Pullman, Washington. June 3-7, 1974.

5. Invited Seminar. Washington State University, Pullman, Wash. "Implications of CO_2 Control of Cell Growth". June 10, 1974.

Publications:

Repaske, R., Repaske, A.C., and Mayer, R.D.: Carbon Dioxide Control of Lag Period and Growth of Streptococcus sanguis. J. Bacteriol., 117: 652-659, 1974.

Serial No. NIAID 62
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Md

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Biochemical Studies on Staphylococcal and Streptococcal Membranes, Cell Walls, and Mesosomal Vesicles.

Previous Serial Number: Same

Principal Investigators: Dr. Eskin Huff and Dr. Theodore S. Theodore

Other Investigators: Dr. Kenneth Nugent
Dr. Gary Calandra
Dr. Roger Cole

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	33/12	-	-	33/12
Professional	21/12	-	-	21/12
Other	12/12	-	-	12/12

Project Description:

Objectives:

To isolate the glycerol containing material found in mesosomal vesicles but not in plasma membranes of Staphylococcus aureus. To determine if this material is glycerol teichoic acid and to determine its structure. To determine if the mesosomes are the sole site for localization of this glycerol teichoic acid. To determine of other Gram-positive organisms have glycerol teichoic acid localized in mesosomal vesicles or in plasma membranes.

Methods Employed:

A new method for the preparation of mesosomal vesicles in increased yields from S. aureus was developed. In this method mesosomal vesicles were obtained from protoplasted cells as has been previously described. In addition

mesosomal vesicles were also obtained from lysed protoplasts and washings of plasma membranes by differential centrifugation. In this manner 80 to 90% of the mesosomal vesicles found in the bacterial cell were recovered.

Standard methods, or previously-described variations (this Project 1973) for gas-liquid chromatography were used. A new method was developed for the isolation of lipoteichoic acid using solvent fractionation. Mesosomal vesicles or plasma membranes of S. aureus and other Gram-positive bacteria were extracted with pyridine-acetic acid-butanol (22:31:100, v/v/v) (PAB) and chloroform-methanol (2:1, v/v) (C/M) to remove lipids followed by treatment with phenol-water (40% final) to yield an aqueous extract containing lipoteichoic acid.

Major Findings:

1. Isolation and chemical characterization of lipoteichoic acid from mesosomal vesicles of Staphylococcus aureus. Mesosomal vesicles of S. aureus were extracted with PAB, C/M, and phenol-water as described above to yield a non-dialyzable compound containing equal amounts of glycerol and phsophate. Partial alkaline hydrolysis of this compound yielded inorganic phosphate, glycerol, glycerol phosphate, glycerol diphosphate, and diglycerol triphosphate as the only glycerol or phosphate containing hydrolysis products. Hydrolysis with 2 N NH_4OH released only alanine (ester linked) and hydrolysis with 2 N NaOH released fatty acids (3% of the dry weight). These results demonstrate that this compound is a poly (glycerol phosphate). The presence of fatty acids indicates further that it is a lipoteichoic acid. The mesosomes contain lipoteichoic acid as a major (18%) component of the dry weight.

A study of the distribution of the intracellular or lipoteichoic acid in various cell fractions of S. aureus showed that 80 to 90% of the lipoteichoic acid in the cell could be accounted for as present in the mesosomal vesicles. Only small amounts of lipoteichoic acid were found in the crude plasma membranes (6%) and periplasm (6%) fraction. Sucrose gradient fractionation removed the remaining lipoteichoic acid from the plasma membranes, and washes of the plasma membrane could be shown to contain mesosomal vesicles. It is clear from these studies that in this organism all of the lipoteichoic acid is in the mesosomal vesicles. (Huff, Theodore, and Cole)

The lipoteichoic acid fraction from mesosomal vesicles and plasma membranes of a number of Gram-positive organisms has been prepared, the glycerol content determined, and the ratio of lipoteichoic acid in the mesosome to the lipoteichoic acid in the plasma membrane determined. These results are reported in project NIAID 63B.

2. Carbohydrate content of group H Streptococcal cell walls and its relationship to sensitivity to phage associated lysin. The cell walls of 10 strains of Streptococcus sanguis (group H), of varying sensitivity to group C streptococcal phage associated lysin (See Project NIAID 65A-4) were hydrolyzed, carbohydrates determined by gas chromatography, and the results

compared to determine any correlation between lysis by the lysin and carbohydrate content. The resistant strains had lowered rhamnose levels, and/or elevated galactose content as compared to the sensitive strains. This correlation between carbohydrate content and resistance to lysis by the phage associated lysin could be of taxonomic importance and of interest in understanding the mode of action of the phage associated lysin. However, more examples will be needed to establish any generality. The resistant strains K 208 and S. sanguis II yielded anhydroribitol (0.3%) as well as ribitol. Cell wall teichoic acid usually yields only ribitol on short term hydrolysis followed by a gradual conversion of ribitol to anhydroribitol over a 3 day period. The presence of anhydroribitol in quantities greater than ribitol in a 1 hour hydrolysis suggests that anhydroribitol occurs as such in the cell wall or that a different type of teichoic acid occurs which hydrolyzes to a mixture of ribitol and anhydroribitol. (Huff, Cole, Calandra, Nugent)

Amino acid analysis of phage resistant strains of Streptococcus group H Wicky have been carried out. Differences in some of the amino acids suggests that structural differences in the murein of the resistant strains prevent action of phage lysis. (Huff, Nugent).

Significance to Biomedical Research and the Program of the Institute:

Our discovery that bacterial mesosomes are the sole or major site for location of the intracellular glycerol teichoic acid or lipoteichoic acid: 1) should bring to the attention of those working with bacterial membranes the difficulty and importance of distinguishing between mesosomal vesicles and plasma membranes; 2) provides for the first time a useful marker which will allow a clear distinction to be made between mesosomal vesicles and plasma membranes; 3) has led us to propose a new hypothesis for control of cell division in which mesosomes, under the influence of DNA accumulate lipoteichoic acid and magnesium ion and thereby become active in localized synthesis of cell wall and septum which leads in turn to cell division. 4) Cell wall analysis of streptococci offers new and adjunct approaches to clarification of taxonomic problems.

Proposed Course of Project:

To test our hypothesis of cell division we propose to : 1) test isolated mesosomal vesicles, broken mesosomal vesicles (methods for which are needed), and plasma membranes for incorporation of labeled precursors into cell wall and teichoic acid in the presence and absence of added lipoteichoic acid and magnesium ion; 2) compare the binding of lipoteichoic acid and magnesium ion to plasma membranes, mesosomal vesicles and broken mesosomal vesicles; 3) determine where lipoteichoic acid occurs within the mesosomal vesicle; and 4) extend cell wall analysis to other groups and strains of streptococci.

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Honors and Awards:

None

Publications:

None

Serial No. NIAID 63B
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on the nutrition, physiology and ultrastructure of Staphylococcus aureus.

Previous Serial Number: Same

Principal Investigators: Dr. Theodore S. Theodore
Dr. Eskin Huff
Dr. Kenneth Nugent

Other Investigators: Dr. Roger M. Cole
Dr. Benjamin Prescott
Dr. Eugene C. Weinbach, LPD (Serial No. NIAID 123C)
Dr. Joseph G. Tully, LID (Serial No.NIAID 207)

Dr. Paul Beining, Dept. of Biology, Univ. of Scranton, Scranton, Pa.

Mr. Dana Twible, Wesleyan University, Middletown, Conn.

Dr. Willie Pachas, Veterans Hospital, Baltimore, Md.

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned To Project	Guest Workers	TOTAL
Total	27/12	-	-	27/12
Professional	15/12	-	-	15/12
Other	12/12	-	-	12/12

Project Description:

Objectives:

To isolate and characterize by cytological and biochemical methods the membrane system of Staphylococcus aureus and other Gram positive bacteria; more specifically, to examine the

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distribution of certain enzymes and other compounds in the membrane system in order to allow for the biochemical, physiological, and structural differentiation of mesosomes and plasma membrane.

Methods Employed:

Routine bacterial and culture procedures; spectrophotometry, polarography, paper and thin layer chromatography, gas-liquid chromatography, polyacrylamide gel electrophoresis and routine metabolic, enzymatic and radioisotope procedures; differential and density gradient centrifugation techniques and electron microscopy.

Major Findings:

1. Localization of lipoteichoic acid in mesosomal vesicles of S. aureus and other Gram positive bacteria.

The localization of lipoteichoic acid almost exclusively in mesosomal vesicles of Staphylococcus aureus ATCC 6538P has been reported (Project No. NIAID 62). This discovery is unique, since up to now lipoteichoic acid of non-wall fractions has been reported only in plasma membranes of Gram positive bacteria. To determine whether the localization of lipoteichoic acid in S. aureus ATCC 6538P mesosomal vesicles was limited to this organism or if it was a generalized phenomenon which occurred in all Gram positive bacteria, we prepared mesosomal vesicles and plasma membranes from several bacteria and analyzed for lipoteichoic acid. S. aureus 80/81, Group A Streptococcus C203S, Bacillus subtilis 168, Bacillus licheniformis ATCC 9945A, Micrococcus lysodeikticus, and Lactobacillus casei var. rhamnosus ATCC 7869 each were protoplasted in appropriate hypertonic buffer with appropriate muralysin and mesosomal and plasma membranes isolated by differential centrifugation. The lipoteichoic acid was fractionated, subjected to acid hydrolysis and phosphatase treatment, and analyzed for glycerol and phosphate. In each bacterium, the concentration of glycerol (μmoles/mg dry weight) was greater in the mesosomal vesicles. The level of glycerol varied by bacterium, and ranged from 4 to 30 fold greater than in the corresponding membrane fraction. The data indicate that the lipoteichoic acid in Gram positive bacteria is associated predominantly with the mesosomal vesicles. (Theodore, Huff, and Cole)

2. Characterization of lipids in mesosomal vesicles.

Lipids of each membrane fraction were extracted with butanol-pyridine-acetic acid and the non-lipid contaminants removed by Sephadex treatment. The lipids were then fractionated on DEAE columns and characterized by thin-layer chromatography, chemical and spectral analysis. The lipids were separated into 4 DEAE

fractions: 1. Vitamin K_2, carotenoids, C55 isoprenoid alcohols, monoglucosyl diglyceride (MGDG); 2. cardiolipin (C), phosphatidyl glycerol (GPG), diglucosyl diglyceride (DGDG); 3. C, GPG; 4. C, GPG, and phosphatidyl glucose (PGL). Qualitatively, no difference in lipid composition between mesosomal and plasma membranes was found; however, based on dry weights and chemical analyses, a relative quantitative difference in the amount of specific lipids in mesosomal vesicles and plasma membranes was observed. The ratios for this quantitative difference in specific lipids from mesosomal vesicles over plasma membranes were: GPG, 3; C, 2.5; MGDG, 3; DGDG, 3; PGL, 3; carotenoids, 2: and vitamin K_2, 1.5. The increased levels of lipids in the mesosomes suggest that these organelles may be sites for de novo membrane synthesis. (Theodore, Beining, and Prescott)

3. Oxidative activities associated with mesosomal vesicles and plasma membranes.

Cytochromes and oxidase activity (terminal and complete sequence of the electron transport chain, respectively) have been shown to be localized primarily in the plasma membranes. Using malate, lactate, succinate and glycerol phosphate as substrates, the dehydrogenase activities (initial sequence of electron transport chain) of plasma membranes was found to be 2 to 9 times greater than that of the mesosomal vesicles. In terminating these studies, it can be concluded that the respiratory chain of S. aureus is associated predominantly with the plasma membrane fraction. (Theodore and Weinbach)

4. Identification of bacterial L forms and mycoplasmas by polyacrylamide gel electrophoresis.

Although Project No. NIAID 65B, "Studies on the identification of bacterial L forms and mycoplasmas by polyacrylamide gel electrophoresis," has been discontinued, an identification service for this class of microorganisms is still maintained. In collaboration with Dr. J. G. Tully (LID) we have aided in the identification and taxonomic classification of several Mycoplasma species of caprine origin; also, we have characterized electrophoretically an unusual cell wall defective organism which may be related to Erysipelothrix rhusiopathiae (causative agent of arthritis in animals). (Theodore, Tully, Pachas)

5. Localization of degradative enzymes.

S. aureus ATCC 6538P was fractionated into plasma membranes, mesosomal vesicles, cytoplasm, periplasm, and culture fluid, and each fraction was assayed for several degradative enzymes. For this summary each activity will be assigned to the particular

fraction that contained the highest specific activity: alkaline phosphatase, acid phosphatase, and 5' nucleotidase in the periplasm; ATPase in the plasma membrane; protease in the mesosomal vesicles; nuclease in the culture fluid. Cell wall lytic activity and esterase activity were not detected in any fraction. S. aureus 80/81 was fractionated in a similar manner after penicillinase induction; this enzyme was most concentrated in the mesosomal vesicles. Ultrastructural analysis did not reveal any morphological changes associated with the secretion of the extracellular fraction of penicillinase. To draw meaningful conclusions from this type of study, the protoplasts must be intact and stable during the early stages of fractionation. Although protoplast stability has been documented in the past by electron microscopy and turbonmetric studies, this question was examined in more detail by following the release of two intracellular markers, glucose-6-phosphate dehydrogenase and deoxyribonucleic acid. More than 89% of these two markers remained in the protoplasts during routine fractionation; this data further documented the stability of these protoplast preparations.

S. aureus 6538P, S. aureus 80/81, and S. aureus strain Wood 46 were used in studies on the intracellular location of Staphylococcal alpha hemolysin. All three strains contained a periplasmic pool of alpha toxin. This periplasmic toxin was recovered both in a soluble form and as a biologically active precipitate during a dialysis step which removed the 20% NaCl used to stabilize the protoplast. These aggregates were stable in hypotonic buffer and contained ribonucleic acid, deoxyribonucleic acid, and protein. This rahter surprising finding has not been investigated further to date.

This project is one of the first broad surveys of degradative enzyme activities in all the cell fractions of S. aureus. The results indicate that the mesosomal vesicles are qualitatively similar to the plasma membranes in degradative enzyme content and that therefore the mesosome plays no special role in these catabolic processes. This data enable us to exclude one postulated function of the mesosome in bacteria, i. e., the mesosome functions as a lysosome-like body. Furthermore, none of the activities studied were suitable as membrane markers for monitoring purification. The demonstration of significant pools of alkaline phosphatase, acid phosphatase, and 5'nucleotidase in the periplasmic space in S. aureus adds to a growing literature investigating the metabolic importance of the periplasm. The surface location (plasma membrane, periplasm) of these various enzymes is relevant to the numerous studies attempting to relate virulence to extracellular enzymes in S. aureus. An invading organism may well be able to utilize host tissue without secreting the degradative enzyme(s) and therefore in vitro assays for strictly extracellular activity need not necessarily correlate with or product virulence.

The studies with alpha toxin are the first to demonstrate an intracellular pool of this toxin. This information should stimulate further research into the cellular metabolism of this important toxin. (Nugent, Theodore, and Cole)

6. Isolation of DNA-membrane complexes.

In preliminary investigations, the Sarkosyl-Magnesium technique for isolating DNA-membrane complexes was adapted to S. aureus. The resulting complexes were isolated from sucrose gradients and chemically analyzed. The average complex contained 81% of the cellular DNA, 38% of the protein, and 26% of the RNA. These figures are comparable to other studies in Gram-positive and Gram-negative organisms. This technique has potential use in isolating a specific small region of the membrane; under carefully controlled conditions 90% of the cellular DNA can be isolated with as little as 4% of the plasma membrane. This portion of the membrane should contain the mesosomal sac remnant. The lipo (glycerol) teichoic acid content would be relevant to current views about cell division.

Significance to Bio-Medical Research and the Program of the Institute:

Our major effort has been directed toward determing what is the function (or functions) of mesosomal vesicles in S. aureus as well as other Gram positive bacteria. Using plasma membranes as a basis for comparison, our approach to this problem has been two-fold: 1) analysis of functional activities purportedly associated with mesosomes; 2) characterization of their chemical composition. At present, the latter approach has yielded the most definitive information. We have succeeded in localizing lipoteichoic acid almost exclusively in the mesosomal vesicles. This polymer, which has been positively identified, consisted of 15 to 20% of the dry weight of the mesosome and was practically absent from all other cell fractions tested (NIAID 62). Also, the lipoteichoic acid has been found to be associated mainly with the mesosomes of six other Gram positive bacteria. This discovery is of great importance, since up to now the lipoteichoic acid of non-wall fractions has been reported only in the plasma membrane fraction of Gram positive bacteria. It has been proposed that the lipoteichoic acid functions as a sequestering agent of Mg^{++} which are needed in high concentrations for the activatior of enzymes involved in membrane and cell wall biosynthesis. Also, we have shown that the same types, but increased levels of lipids are present in mesosomal vesicles which suggests that these organelles may be the sites of membrane biosynthesis. Lastly, it has been found that penicillinase activity of a penicillin-resistant strain of S. aureus is associated with mesosomes. Although significant differences in the distributions of degradative enzymes between membrane and mesosome was not found. If one takes into account all of these functional activities and associates them with a

specific organelle, the mesosome would be the most likely candidate, since, these organelles arise from the membranous septum and precede the formation of crosswall. Hence, mesosomes would appear to play not only a vital role in membrane and cell wall biosynthesis, but also as a protective site against antibiotic attack.

Proposed Course of Project:

We hope to pursue the following lines of investigation:

1. The complete chemical analysis of lipoteichoic acid and the localization and assay of the enzymes involved in the biosynthesis of this polymer. The participation of mesosomal vesicles and plasma membranes and the distribution of enzymes pertaining to membrane and cell wall biosynthesis of Gram positive bacteria.

2. Determination of serological activities of glycolipids and lipoteichoic acid isolated from Staphylococcus aureus.

3. Attempts at the histochemical localization of lipoteichoic acid in Gram positive bacteria.

4. Amino acid analysis of mesosomal vesicles and plasma membranes.

Honors and Awards:

Representative of NIAID to Interassembly of Scientists Council.

Publications:

Theodore, T. S., and Panos, C.: Protein and fatty acid composition of mesosomal vesicles and plasma membranes of Staphylococcus aureus. J. Bacteriol. 116: 571-576, 1973.

Tully, J. G., M.F. Barile, D. G. ff Edwards, T. S. Theodore, and H. Ernø: Characterization of some caprine mycoplasmas, with proposals for new species, Mycoplasma capricolum and Mycoplasma putrefaciens. J. Gen. Microbiol. (In press)

Theodore, T. S., and Weinbach, E. C.: Respiratory activities associated with mesosomal vesicles and protoplast membranes of Staphylococcus aureus. J. Bacteriol. (In press)

Serial No. NIAID 63C
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Reports
July 1, 1973 through June 30, 1974

Project Title: Investigation of Gram-Positive Cocci

Previous Serial Number: Same

Principal Investigator: Dr. Arthur L. Schade

Other Investigators: None

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	20/12	-	-	20/12
Professional	12/12	-	-	12/12
Other	8/12	-	-	8/12

Project Description:

Objectives:

To effect the isolation, purification, and characterization of the type-specific M protein antigen from the difficultly typable strain of Streptococcus pyogenes, type 4, whose frequency of identification as the etiologic agent in cases of human streptococcal disease is relatively and significantly high.

Methods Employed:

Standard methods of bacteriology, metabolism, and biochemistry. Special methods, as required, include a variety of immunological procedures; radioactivity techniques; spectrophotometry; electrophoresis; chromatography; and ultracentrifugal gradient separations. Specifically, the following methods for isolation of M protein were developed:

Streptococcus pyogenes. M-type 4 (strain CDC-0107) cultures were grown 3-4 hrs under 95% N_2 + 5% CO_2 at 37 C with constant moderate stirring and automatic pH control at 7.4 in double strength Todd-Hewitt broth at 1% glucose and cysteine. HCl to 0.0014 M. Cells were washed 3 times with phosphate-buffered saline (PBS), pH 7.0 and extracted with molar LiCl in 0.25 M acetate buffer at pH 4.4 for 48 hrs at 37 C. The supernatant was positive for M protein with CDC type 4 antiserum and, following vacuum dialysis, the concentrated retentate was dialyzed vs. acetate buffer at pH 4.7. A precipitate formed containing all but traces of the M reactive material. G100-Sephadex gel filtration at pH 7.0 separated the active fraction which was subsequently precipitated with 60% ammonium sulfate. Chromatography on a Cellex ion-exchange column at pH 4.83 gave the reactive material only in the void volume with 0.01 M acetate as eluant. Freezing and thawing at pH 4.83 precipitated the active material, now labelled "A" and available for tests summarized below.

The LiCl ultrafiltrate from vacuum dialysis was 60% ammonium sulfate precipitated and the flocculent material was redissolved in phosphate buffer at pH 7.0 to give a strong positive reaction vs. M antiserum. The M-reacting solution was dialyzed vs. 0.01 M acetate buffer, pH 4.55 and chromatographed on to a CM-Sephadex column. Following gradient elution, a peak of activity appeared when the resistance was lowered to ca. 200r and the pH had risen from 4.55 to ca. 6.0. For tests this material is designated "B".

Major Findings:

Tests and results:

1. Preliminary opacity tests by the tube method of Widdowson et al were strongly positive for culture broth supernatants but equivocal for the final fractions "A" and "B". The agar slide method gave dubiously replicable results.

2. Ouchterlony plates using the CDC specific antiserum vs "A" produced 5 lines at the highest concentration employed. "B" gave 2 lines, one of which was continuous with one of the 5 in "A".

3. 0.01 ml of each "A" and "B" failed by the Bendisch fluorescent chromatographic test to give any evidence of nucleic acid bases at levels of at least 0.025 µg of either guanine or xanthine. The size of sample may well be limiting.

4. Subjection of "A" and "B" to alkaline acrylamide gel electrophoresis resolved 7 distinct lines for "A" and 13 components for "B" for the amounts analyzed. Acid gel electrophoresis revealed no lines for "A" and at least one for "B".

Conclusions from these experiments include:

1. LiCl extraction of streptococcal cells releases at least 5 materials reactive with the CDC "M" specific antiserum.

2. At least two extractable reactive components are dialyzable under vacuum dialysis and one or more are dialyzable under passive conditions.

3. More protein-staining materials than appear to be precipitable by the antiserum are separable on acrylamide alkaline gel through electrophoresis; at least one dialyzable component bears a strong basic character as revealed by electrophoresing on acid gel.

4. Which of the antiserum-precipitable components is the "M" protein of virulence-factor capacity is not known but could be determined. How the electrophoretic components relate to the antiserum precipitable ones is also unknown. It appears probable that the opacity factor may be separable from at least some of those components that react with the antiserum.

Significance to Bio-Medical Research and the Program of the Institute:

Currently there are known at least 60 specific types of Streptococcus pyogenes, each characterized by possessing an immunogenically distinguishable surface layer protein, its M protein. As McCarty has observed, not only are the M proteins necessary for the virulence of these organisms "but they serve as the determinant of the prevalent pattern of human streptococcal disease." Lacking adequate knowledge of the structure of these proteins and having "none on the nature of that common feature of this serologically diverse family of antigens that confers the property of resistance to phagocytosis," we shall advance bio-medical research by realizing the objectives of this project. Since type 4 Group A streptococci are frequently identified as etiologic agents in cases of human disease, the successful purification and characterization of its M protein would allow its inclusion in a polyvalant vaccine and enhance its prospective efficacy. Such a result will help to fulfill the program of the Institute, and its interest in streptococcal disease as a special emphasis area.

Proposed Course of Project:

Successful prosecution of the project to isolate and characterize the "M" protein of type 4 Streptococcus at this juncture calls for large scale production of cells, LiCl extraction followed by either the ammonium sulfate precipitation of active

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components or vacuum dialysis and concentration of the extract with salt precipitation of the dialysate, and use of preparative acid and alkaline acrylamide gel electrophoresis of the products to separate further the components. The isolated and concentrated fractions should then be subjected to all the definitive tests to establish any final product as the physiochemically pure and biologically active "M" protein.

Honors and Awards:

At the forthcoming meeting of the ASM in Chicago, a symposium on, "Roles of Iron in Host-Parasite Interactions," is to be dedicated to me and my assistant of many years ago for opening up and carrying on a field of research of obvious interest to Medical Microbiology. I have prepared a short original paper as my acknowledgement of this honor.

Publications:

None

Serial No. NIAID 64-C
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on mycoplasmal, plant and mammalian tissue glycolipids.

Previous Serial Number: Same

Principal Investigator: Dr. Benjamin Prescott

Other Investigators: George Caldes

Cooperating Units: Laboratory of Infectious Diseases (NIAID 70C)
Dr. Robert M. Chanock
Dr. Harry Greenberg
Dr. Charles M. Helms
Walter James

Man Years:	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	12/12	-	-	12/12
Professional	6/12	-	-	6/12
Other	6/12	-	-	6/12

Project Description:

Objectives:

Isolation, purification and characterization of serologically active glycolipids from plants and brain tissue that cross react with rabbit and human antiserum to Mycoplasma pneumoniae, as homogeneous substances for use in immunogenic studies.

Methods:

Fractionation methods used for the isolation and purification of the plant lipids into individually serologically active molecular structures was

achieved by separation with various mixtures of organic solvents by methods previously described (64C 1972).

New methods for brain lipid antigens were as follows: Human brain tissue obtained at autopsy washed with water to remove blood was homogenized in chloroform-methanol (CM) (2:1 v/v) 10 ml/g. of tissue. Held at room temperature overnight and filtered through a No. 50 Whatman paper on a Buchner funnel and freed from water soluble substances by solvent partition with 0.1M K Cl in water. The chloroform layer was concentrated in vacuo under nitrogen. Preliminary separation of cerebrosides was carried out by column chromatography on alumina and eluted successively with (1) CM (19:1 v/v), (2) CM (1:1 v/v); (3) CM-H_2O (7:7:1); and (4) Ethanol-chloroform-water (5:2:2). The fractions were analyzed for carbohydrate to determine which fraction contained the glycolipids (cerebrosides). Fraction 3 contained almost all the cerebroside present in the original lipid extract. The cerebroside was determined by analysis for combined galactose. Further treatment of this fraction was carried out by thin layer chromatography with slightly modified procedures of those already described (64D) 1974.

Major Findings:

Plant: Galactose-rich glycolipids appear to have a wide distribution in plant materials as shown in our previous studies (64C 1972). These compounds were shown to cross-react with antibodies to Mycoplasma pneumoniae in the complement-fixation test. Mild alkaline Hydrolysis of an active lipid hapten (isolated from carrot)with 0.1M KOH in methanol gave the following components, galactose-fatty acid ester-glycerol in a ratio of 2.01:2.00:1:00. Since this ratio is found with commercially purified spinach digalactosyl diglyceride, the molecular composition of the serologically active carrot glycolipid seems to be structurally related to the widespread antigenic lipid digalactosyl diglyceride present in numerous plant substances. In addition, enzymic hydrolysis with galactostat degraded the intact glycolipid structure and thin-layer and paper chromatography of the products indicated that the active carrot glycolipid hapten has the structure of a digalactosyl diglyceride. Analytical analysis of the purified serologically active lipid hapten showed the following concentrations of the components; 37% galactose; 9.5% glycerol; 64.0% fatty acid methyl esters. The analysis also suggests high concentrations of dienoic and trienoic acids coupled by ether linkages. The water soluble deacylation product of the hapten and the hexane soluble fatty acids were without activity when tested by complement fixation with M. pneumonia antisera. Confirmation of the linkages between the units in the lipid hapten provided evidence as to the relative position of the carbohydrate components and fatty acid molecules. The molar ratios were calculated for a digalactosyl diglyceride with linoleic acid as the fatty acid, $C_{57}H_{94}O_{15}$ with a molecular weight of 946.

Brain: At the same time that the work on the plant glycolipids was in progress, studies were also being carried out on the isolation and characterization of lipid haptens from human brain tissue since it has been shown that extracts of brain tissue react with antibodies to Mycoplasma pneumoniae in the complement-fixation test.

The crude brain lipid extracts showed a lipid spot on chromatograms similar to one present in M. pneumoniae and plant lipid extracts. It was ninhydrin-free and had the staining properties of a glycolipid, periodate-Schiff reagent on thin layer plates. The purified lipid scraped from thin layer chromatograms was homogeneous. Analytical analysis showed the compound to contain galactose, glycerol and fatty acid ester in a molar ratio of 1.00:1:00:2.08. Preliminary evidence suggests that the serologically active material is a galactosyldiglyceride. Mild hydrolysis, hydrogenation or periodate treatment of the lipid hapten destroys its complement-fixation activity, indicating that the activity may be related to the antigenic determinant (galactose) and thus to the stereospecificity of the molecular configuration of the hapten.

Significance to Biomedical Research and the Program of the Institute:

Brain: In certain individuals with Mycoplasma pneumoniae infections, there develop neurological complications. The possibility exists that certain lipids with antigenic activity similar to the active lipid of M. pneumoniae or plant materials may be present in the host and thus may be a factor in the cause of the neurologic phenomena. At present not much is known about the possibility of a pathogenic role of M. pneumoniae-induced antibodies reacting with brain or other tissue antigens. However, possibilities should be considered that within the large number of cross-reacting antibodies there may exist antibodies with cytotoxic activity that could damage brain, nerve or other tissues.

Lipid antigens in brain tissue that cross react with M. pneumoniae antibodies in the CF test contains an antigenic determinant similar to a lipid antigen of M. pneumoniae. The result of our studies demonstrate that there may be a wide distribution of lipid antigens that cross react with M. pneumoniae antibodies among other tissues (lung).

Proposed Course of Project:

An intensive study is in progress to isolate, purify and determine the molecular configuration of additional serologically active glycolipids in plant materials and tissue sources that cross react with M. pneumoniae antisera. Attempts to produce an immunogenic product with these glycolipid haptens for use as a potential vaccine and immunological studies will be intensified.

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Honors and Awards:

Consultant to NIH Library on purchases of certain chemical journals.

Consultant to Vaccine Development Committee, Infectious Diseases Branch, Collaborative Research Division.

Publications:

Barden, J.A., and Prescott, B.: Chemical and serologic properties of Mycoplasma hyorhinis fractions. Infection and Immunity 7: 937-940, 1973.

Greenberg, H., Prescott, B., Brunner, H., James W., and Chanock, R.M.: Sharing of glycolipid antigenic determinants of Mycoplasma pneumoniae. Vegetables and certain bacteria. Monograph of Workshop on New Approaches for Inducing Natural Immunity to Pyrogenic Organisms. (In press)

Prescott, B., King, M.L., Caldes, G., Li, C.P., Young, A.M.: Characterization of an antitumor substance from clam (Mercenaria mercenaria) liver. Int. J. Clin. Pharmacol. 9: 1-5, 1974.

Serial No. NIAID 64D
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Isolation, separation, and characterization of antigenic lipid hapten(s) from streptococci.

Previous Serial Number: Same

Principal Investigator: Dr. Benjamin Prescott

Other Investigators: George Caldes

Cooperating Units: Laboratory of Parasitic Diseases, NIAID
Dr. Theodore E. Nash
Dr. Franklin A. Neva (Serial No. NIAID 121-K)

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	28/12	-	-	28/12
Professional	16/12	-	-	16/12
Other	12/12	-	-	12/12

Project Description:

Objectives:

The isolation, purification, and characterization of serologically-active glycolipids from Streptococcus pyogenes 64x392 (serogroup A) and Streptococcus faecium F24-L (serogroup D); and studies on the cross-reactivity of these derivatives with streptococci and other organisms.

Methods Employed:

The isolation and column chromatography procedures were essentially the same as previously described (64C, 1969-70) with the exception of one new solvent system capable of separating the phosphoglycolipids from other phospholipids. The existence of more than one serologically active glycolipid in Groups A and D streptococci was demonstrated in our previous studies (64D, 1972). By means of preparative thin-layer chromatography of the carbohydrate-rich CM 7:3 fraction from DEAE-cellulose, 5 subfractions

consisting predominantly of one major and two minor antigenic glycolipids and two inactive fractions were isolated. A lack of knowledge about the chemical nature of the two minor antigenic lipid haptens in streptococci turned our investigations to the isolation, purification and characterization of these compounds. One of the serologically active lipids was found to contain phosphorus. Most of our studies reported here were carried out with this fraction because of the higher yields.

Column Chromatography:

Lipid extraction of Groups A and D streptococci was carried out with a mixture of chloroform-methanol-acetic acid-water for 18 hours at room temperature. The lipid extracts were filtered and the filtrates freed of non-lipid contaminants with 0.1 M potassium chloride. Fractionation of the crude extracts was carried out on columns of DEAE-cellulose followed by rechromatography of CM 7:3 carbohydrate-rich lipid fraction on silicic acid with chloroform, acetone, and chloroform-methanol (1:1). The serologically active lipid hapten(s) was purified and concentrated in the acetone fraction.

Thin-layer chromatography:

The following methods were used to establish identity and homogeneity: Thin layer (500μ) Silica gel G glass plates (20x20cm) with 1% sodium borate were activated at 110° C for 30 minutes prior to two-dimensional development first in chloroform-methanol-acetic acid-water (170:25:25:6) and then in chloroform methanol - 7N - NHOH (65:30:4). Plates were dried between the solvent developments with nitrogen at room temperature for 30 minutes to minimize lipid degradation. The chromatograms were dried in an oven at 80° C for one hour cooled and sprayed with the molybdate reagent of Dittmer and Lester for phospholipids and with periodate-Schiff reagent for vicinal hydroxyl groups. Pooling of lipids from unstained plates was necessary for the assay of the different lipids. Neutral lipids were further resolved by one-dimensional thin-layer chromatography in chloroform-methanol-water (65:25:4) and thereafter proved to be homogenous. Identification of the minor antigenic lipid haptens was carried out by acid and alkaline hydrolysis.

Analytical procedures:

The analytical methods were essentially the same as previously described 64D (1969-72). Deacylation of the phosphoglycolipids was performed by shaking a sample (4ml) of the material with 0.4M methanolic KOH (1ml) for 15 minutes. Dowex ($50H^+$) and water (4ml) added, shaken and filtered through a sintered glass funnel and filtrate neutralized. The water phase was evaporated to dryness and the chloroform phase washed with water in a separatory funnel, dried in vacuo and used for analysis of fatty acids.

Major Findings:

The complex lipid extracts from streptococci were purified by column chromatography in combination with thin-layer chromatography. The purified phosphoglycolipids isolated from Group A and D streptococci were

chromatographically identical and contained d-glucose, glycerol, fatty acid ester, and phosphate in a molar ratio of 2:2:2:1. These compounds showed high titers in the complement-fixation test with rabbit hyperimmune sera to whole cells of streptococci (Groups A and D) and of A. laidlawii. Thus the molecular configuration of the antigenic determinants are similar in the serologically active lipids of group A and D streptococci and A. laidlawii. For structural analysis the phosphoglycolipids were deacylated under mild alkaline hydrolysis. The water soluble product contained all the lipid glycerol, glucose and phosphorus in a molar ratio of 2.00:2.02:1.00, which indicates that the lipid contains only ester bonds.

Strong alkaline hydrolysis (N-NaOH at 100°C for 3 hours) completely splits the compound into glycerophosphate and a phosphate-free, carbohydrate-containing compound. The phosphate-free compound showed glucose and glycerol in molar proportions of 2:00:1.00. This compound had an optical rotation ($\alpha_D^{20^\circ}$) of 62.5° and was hydrolyzed by glucostat (β-glucosidase). The phosphorus containing compound extracted from the strong alkaline hydrolysate was identified by chromatography and analysis as glycerophosphate (glycerol, P-vicinal hydroxyls) 1.00:1:00:0.96 respectively. Oxidation of the intact lipid with periodate in chloroform-ethanol-water (2:3.5:1.5) produced no formaldehyde, suggesting that the glycerols contain fatty acids in the molecule. The molecular composition of the serologically polar antigenic phosphoglycolipid isolated from streptococcal groups A and D shows the composition, chromotographic properties and behavior on alkaline hydrolysis to be that of a glyceryl phosphoryl diglucosyl diglyceride.

Reaggregation:

Continued studies with rabbit sera of reaggregates of streptococcal glycolipids and A. laidlawii membrane proteins showed that sera from the second and third bleedings of rabbits immunized with the reaggregate gave high titers in the complement fixation test with the reaggregate glycolipid and whole cell vaccine (64D, 1972-73).

During collaborative studies with Drs. Nash and Neva of the Laboratory of Parasitology on the isolation of a circulating antigen of Schistosoma mansoni, the lipids of the worms were also studied for their potential antigenic activity. In preliminary experiments, it was observed that there are glycolipids present in the worms which cross react with rabbit hyperimmune streptococcal antisera (Groups A and D) by complement-fixation; and that the streptococcal glycolipids cross-react with rabbit anti-schistosome) sera. Thus, there seems to be an antigenic identity between haptens of the glycolipids of streptococci groups A and D and of schistosomes.

Significance to Biomedical Research and the Program of the Institute:

The phosphoglycolipids isolated from groups A and D streptococci represent a new class of glycolipids that possess serological activity in complement-fixation tests. Chemical and physical methods have been employed that facilitate purification and concentration of this active material. Streptococci represent a potential source of phosphoglycolipids which, when purified, prove to be valuable in the characterization of the active lipid

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hapten. Analytical studies showed the structure of the compound to be a glyceryl phosphoryl diglucosyl diglyceride. These antigenic compounds are very useful in studies on the relationship between structure and biological activity, and in the preparation of modified and synthetic antigens by reaggregation with membrane and other proteins which do not affect the antigenic activity but increase immunogenicity.

Proposed Course of Project:

Emphasis will be given to current concepts regarding chemical, physical, and biological properties of the lipid haptens. Further studies will be directed toward the preparation of additional reaggregates. It may be possible to reaggregate the lipid haptens with materials like toxoids, thus introducing bifunctional characteristics in the protein component of the reaggregate. Properties of new derivatives of this type may enhance the possibility of locating the active sites in the reaggregate.

Honors and Awards:

Continuing membership:

Toxicology Information Program (TIP) at the National Library of Medicine. On roster of Authorities in Specialized Subtopics in Toxicology.

Consultant to Inventions Office, DHEW.

Publications:

Caldes, G., and Prescott, B.: A comparative study of certain aniline salts used in the determination of carbohydrates by spot elution chromatography. J. Chromatography 84: 220-223, 1973.

Nash, T. E., Prescott, B., and Neva, F. A.: The characteristics of a circulating antigen in Schistosomiasis. J. Immunol. 112: 1500-1507, 1974.

Feinman, S. Birnbaum, Prescott, B., and Cole, R. M.: Serological reactions of glycolipids from streptococcal L forms. Infection and Immunity 8: 752-756, 1973.

Serial No. NIAID 65A-1
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Group H streptococcal bacteriophages: effects on genetic systems of transformable streptococci.

Previous Serial Number: Same

Principal Investigator: Dr. C. Lowell Parsons

Other Investigators: Dr. Roger M. Cole
Miss Alba E. Colon
Mrs. Carmen G. Leonard
Dr. Jon M. Ranhand

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	-	-	-	-
Professional	-	-	-	-
Other	-	-	-	-

Project Description:

See 1973 Annual Report.

Project has been terminated.

Serial No. NIAID 65G
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Characterization of biophysical and physiological properties of L forms.

Previous Serial Number: Same

Principal Investigator: James R. King

Other Investigators: None

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	-	-	-	-
Professional	-	-	-	-
Other	-	-	-	-

Project Description:

See 1973 Annual Report.

Project has been terminated.

Serial No. NIAID 79-B-1
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Host response to pathogenic fungi in neoplastic disease and immunosuppression.

Previous Serial Number: Same

Principal Investigator: H. F. Hasenclever

Other Investigators: None

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	-	-	-	-
Professional	-	-	-	-
Other	-	-	-	-

Project Description:

See 1973 Annual Report.

Project has been terminated.

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Serial No. NIAID 79C-1
1. Microbiology
2. Medical Mycology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Ecologic and Aerobiologic studies of Histoplasma capsulatum

Previous Serial Number: Same

Principal Investigator: H.F. Hasenclever

Other Investigators: None

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	5/12	-	-	5/12
Professional	4/12	-	-	4/12
Other	1/12	-	-	1/12

Project Description:

Objectives:

To study the factors that contribute to the presence or absence of Histoplasma capsulatum in the soil.

Methods Employed:

The site under study, where H. capsulatum is known to have been present for years, is a farm bat roost at Clarksburg, Md. The other site, studied under this project for the past six years at 7th St. and Penn. Ave., can no longer be investigated because of construction of the subway system.

Soil samples are collected at intervals and the presence or absence of the fungus is determined by mouse inoculation.

Major Findings:

Although no collections of soil were made at 7th St. and Penn. Ave., the percentages of positive isolations were compared from 1967-1972. These ranged from 2.5% to 9%. A similar comparison was made at Clarksburg. The percentage of positives from soil samples collected randomly over the site from 1968-1973 ranged from 4-8%. In a small area that in 1969 was found to be strongly positive (48%), 1972 (13%), and 1973 (4%).

At the site in Clarksburg a soil culture of H. capsulatum was added to 0.5 sq. m. that had not been previously fertilized with bat guano and to 1.0 sq. m. that had been fertilized with approximately 1 kg of bat guano divided into six applications over a three year period. Soil samples collected from the fungus seeded but unfertilized area five months later were 4/4 positive, ten months later 0/4, and eleven months 0/4. Samples collected from the seeded fertilized area six weeks after seeding were 10/12 positive, seven months 6/8 positive, twelve months 2/6 positive, and thirteen months 2/6 positive. Collections made eighteen months after seeding from both locations are now being processed.

Results from this project indicate that for a period of approximately five years after the natural fertilization of soil by birds or bats, colonization of the site by H. capsulatum is heavy but then diminishes and the soil samples remain from 2.5-9% positive for at least another five years.

Significance to Biomedical Research and the Program of the Institute:

Information on the longevity and factors contributing to the continued existence of H. capsulatum in its natural state is being accumulated. This may eventually permit the development of a rational biological control of the fungus in nature.

Proposed Course of Project:

To be terminated because of disestablishment of the Laboratory of Microbiology.

Honors and Awards:

None

Publications:

Hasenclever, H.F., and Piggott, W.R.: Colonization of soil by Histoplasma capsulatum: I. Factors affecting its continuity in a given site. Health Laboratory Science (In press)

Serial No. NIAID 79D
1. Microbiology
2. Medical Mycology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Immunochemical Studies on Polysaccharides from Fungi

Previous Serial Number: Same

Principal Investigator: H.F. Hasenclever

Other Investigators: Errol Reiss

Cooperating Units: John Ellsworth, Guest Worker, George Washington University

Man Years:	NIAID Lab. Staff	Other Personnel Assigned to Project	Guest Workers	TOTAL
Total	27/12	-	-	27/12
Professional	16/12	-	2/12	18/12
Other	11/12	-	-	11/12

Project Description:

Objectives:

To study (1) antigenic relationships within or among species of yeasts; and (2) the molecular structure, serologic and immunobiologic properties, and the haptenic groups of polysaccharides or other antigens isolated from yeasts and fungi.

Methods Employed:

The serologically reactive substances are extracted by carefully controlled methods from fungal cells or isolated from the culture media in which the fungi were grown. Serologic similarity or diversity are shown by agglutination, immunodiffusion, or quantitative precipitation. Biologic activity is shown by injection in hypersensitive animals. The monosaccharide content of polysaccharides is determined by gas chromatography. Antisera for serologic studies are prepared by injection of rabbits with fungal cells.

Major Findings:

Studies comparing the serologic relationship of serotypes A, B, and C of Saccharomyces telluris with Candida albicans antigenic Groups A and B and S. cerevisiae by agglutination and immunodiffusion have been conducted. Results indicate that S. telluris serotype A and C. albicans Group A are the most closely related. Although as shown by agglutination, the serotypes of S. telluris share a common serologic activity not found in C. albicans A, the mannans from S. telluris A and C. albicans A share common major haptenic groups not present in mannans of the other two serotypes of S. telluris or C. albicans A and B and S. cerevisiae share serologic properties not found in any of the serotypes of S. telluris. Agglutination appears to be more sensitive in showing minor antigenic differences among the yeasts studied than using mannans in immunodiffusion tests.

A galactomannan-glycoprotein complex extracted from defatted mycelium of Histoplasma capsulatum has been further characterized. The polysaccharide and the glycoprotein were separated by DEAE cellulose chromatogrpahy. The galactomannan formed a single precipitin band by immunodiffusion and did not elicit a skin test in hypersensitive guinea pigs, but 10 ug resulted in 80% inhibition of macrophage migration. The glycoprotein produced a delayed hypersnesitivity reaction in sensitized guinea pigs, and 5 ug resulted in 86% inhibition of macrophage migration.

A cytoplasmic protein has been isolated from mechanically disrupted yeast cells of C. albicans that reacts in immunoelectrophoresis with sera from infected rabbits and patients with chronic candidiasis. This protein elicits delayed hypersensitivity in sensitized guinea pigs and contains no detectable mannan.

A soluble peptidoglucomannan (PGM) and a mannoglucan (sMG) have been extracted from cell walls of C. albicans. The insoluble residue of the cells walls has been characterized as a mannoglucan (iMG). The amino acids of the PGM have been identified and constitute 7.3% of the total material. The glucomannan of the PGM has a glucose to mannose ratio of 2:3. The sMG has a glucose to mannose ratio of 6:1. The insoluble mannoglucan residue contains 11% mannose and is agglutinated by antiserum to whole C. albicans yeast cells. The antigenic group difference of C. albicans A and B is observed by immunodiffusion with both the mannan content of each. The PGM, sMG, and iMG all specifically inhibit the migration of machrophages.

Significance to Biomedical Research and the Program of the Institute:

These results are showing the serologic relationships that exist among pathogenic and nonpathogenic yeasts and are defining antigens that provoke antibody responses and induce delayed hypersensitivity in some fungal disease.

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Proposed Course of Project:

To be terminated because of disestablishment of the Laboratory of Microbiology.

Honors and Awards:

Chairman, Standards and Examining Committee for Certification in Medical Mycology, American Board of Medical Microbiology.

Publications:

Reiss, E., Mitchell, W.O., and Hasenclever, H.F.: Isolation and fractionation of an antigenic galactomannan-protein complex from whole cells of the fungus, Histoplasma capsulatum. Infection and Immunity (In press).

Reiss, E., Stone, S.H., and Hasenclever, H.F.: Serologic and cellular immune activity of pepido-glucomannan fractions of Candida albicans cell walls. Infect. Immun. (In press).

Serial No. NIAID 79E
1. Microbiology
2. Medical Mycology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Yeast Flora of Birds

Previous Serial Number: Same

Principal Investigator: H.F. Hasenclever

Other Investigators: None

Cooperating Units: Dr. R.M. Kocan
Patuxent Wildlife Research Center

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	4/12	-	-	4/12
Professional	4/12	-	-	4/12
Other	-	-	-	-

Project Description:

Objectives:

To study qualitatively the yeast flora of columbiform birds with particular emphasis on Columba livia (common pigeon); and to determine the frequency of isolation of those microorganisms that are associated with and cause disease in humans.

Methods Employed:

Pigeon droppings were collected at rural and urban sites and cultured for C. albicans.

Major Findings:

C. albicans was isolated from 11 of 34 samples of pigeon droppings. The positive samples contained from 10^2 to 6 x 10^5 colony forming units of C. albicans/gm of dry feces.

Significance to Biomedical Research and the Program of the Institute:

These results extend our knowledge concerning the ecological relationship of C. albicans with the common pigeon. This bird, being a close associate of man and his architecture, may represent a source of contagion for this yeast.

Proposed Course of the Project:

To be terminated because of disestablishment of the Laboratory of Microbiology.

Honors and Awards:

None

Publications:

Kocan, R.M., and Hasenclever, H.F.: Seasonal variations of the upper digestive tract yeast flora of feral pigeons. J. Wildlife Dis. (in press)

Serial No. NIAID 152
1. Microbiology
2. Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on antibody production and mechanisms of hypersensitivity

Previous Serial Number: Same

Principal Investigator: Dr. Sanford H. Stone

Other Investigators: None

Cooperating Units:

1. Dr. Marius Valsamis
   Dr. Cedric Raine
   Division of Neuropathology
   Albert Einstein College of Medicine
   New York, New York

2. Dr. Gardner Middlebrook
   Dr. Zulema Reggiardo
   Division of Experimental Pathology
   University of Md. School of Medicine
   Baltimore, Maryland

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	18/12	-	-	18/12
Professional	12/12	-	-	12/12
Other	6/12	-	-	6/12

Project Description:

Project Subtitle I: Mechanisms of protection against Mycobacterium tuberculosis (in collaboration with Cooperating Unit #2)

Objectives:

To elucidate the mechanisms underlying protection against tuberculosis in guinea pigs.

Methods Employed:

Inbred histocompatible guinea pigs were sensitized by inoculation of living BCG vaccine or infection of killed M. tuberculosis in oil emulsion. Lymph node or spleen cells were taken during various stages of the immune response and transferred to histocompatible and random bred recipients. Recipients were challenged by aerosols containing living M. tuberculosis. Organs were examined for the presence of M. tuberculosis and its lesions by bacteriological and histological criteria.

Major Findings:

From animals systemically immunized with living BCG, lymphoid cells do not transfer significant delayed hypersensitivity to tuberculin, but can under appropriate conditions (involving donor-recipient ratios, time of harvest, etc.) adoptively transfer protection against challenge by aerosols containing virulent M. tuberculosis. Guinea pigs immunized with killed M. tuberculosis in water-in-oil emulsion were not suitable donors for such adoptive protection, and recipients of their lymphoid cells had lesions comparable to controls.

Significance to Bio-Medical Research and the Program of the Institute:

Although the search for better vaccines for tuberculosis has occupied the attention of investigators for many decades, the actual mechanisms of protection against this disease remain unexplained. The elucidation of the components of the immune response involved in protection would lead to better choices of form of antigen and/or adjuvant. Passively transferable mechanisms of importance in therapy of other diseases (tumors, leprosy) also require clarification.

Proposed Course of Project:

Appropriate methods for preparing purified suspensions of B cells and T cells will be adopted for use in experiments on transfer of protection against tuberculosis. The role of macrophages from both immunized and normal hosts will be examined.

Project Subtitle II: Genetics and pathogenesis of acute and chronic autoimmune encephalomyelitis and autoimmune neuritis

Objectives:

To elucidate the mechanisms underlying resistance to autoimmune encephalomyelitis (AE) linked to age, sex, or strain differences in guinea pigs, in search for information on the

etiology of the disease or of demyelinating diseases in general. (In collaboration with Cooperating Unit #1)

Methods Employed:

Young and mature, male and female strain 2 and strain 13 guinea pigs were sensitized with homologous spinal cord or peripheral nerve tissue incorporated into Freund's complete adjuvant (FCA) containing killed Mycobacterium tuberculosis. The effects of subsequent active or passive immunization with the same antigen were observed. Tissues from guinea pigs with acute, sub-acute, and chronic AE were taken for detailed comparison of these conditions using classic histological methods and analyses by electron microscope (EM) as well. Treatment of chronic AE, which resembles multiple sclerosis, was undertaken with the immunosuppressive drug cytoxan injected intraperitoneally.

Major Findings:

In contrast to adult strain 13 guinea pigs which always develop acute AE, juvenile animals develop a more chronic disease, which shows (after clinical signs have appeared) morphological evidence of both recent damage to the myelin and remyelination. This suggests an earlier subclinical disease process. Ultrastructural examination revealed repeated acute episodes of demyelination: spinal cords became infiltrated by Schwann cells which myelinated previously denuded CNS fibers. The chronic disease could not be transferred by lymphoid cells alone to adult or weanling recipients.

Significance to Bio-Medical Research and the Program of the Institute:

This chronic age-dependent AE is highly reproducible and bears many morphologic and clinical resemblances to multiple sclerosis. The similarities to MS plaques in the CNS and the chronicity with relapse and remission make this form of AE a highly relevant animal model for demyelinating diseases in man.

Proposed Course of Project:

T and B cells from guinea pigs with chronic AE will be used in vitro in tissue culture of myelinated CNS tissues and in vivo alone or in combinations to correlate particular immune responses with the various types of chronic and acute AE.

Project Subtitle III: Studies on cellular manifestations of hypersensitivity and immunity in guinea pigs: transplantation of immunologically competent tissues

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Objectives:

Correlation of the developments in lymphoid tissues draining the sites of injection of antigens with the capacity of the lymph node T or B cells to transfer either hypersensitivity or antibody-producing capacity.

Methods Employed:

Immune deviation (suppression of manifestation of delayed hypersensitivity) was established by injection of alum-precipitated antigens in guinea pigs. In appropriate passive versions of the model, guinea pigs were sensitized with various antigens incorporated into complete or incomplete Freund's adjuvant or in alum adjuvants. They were used as donors or recipients of lymphoid cells, in order to isolate and characterize the role of the adjuvant in selecting components of the immune response.

Major Findings:

Adoptive transfer of immune deviation was not easily achieved with lymph node cells either from deviated donors or from deviated and sensitized donors. Peritoneal exudates appear to be more suitable for such transfers.

Significance to Bio-Medical Research and the Program of the Institute:

The types of cells required for transfer of adoptive immunity are of interest for the reconstruction of the immune response at the cellular level. Which cells are susceptible to immune deviation and also the mechanism of immune deviation may be queried by this approach. The adoptive transfer is a powerful tool for study of the cell-cell interactions recently recognized of great importance in immune responses. The importance of the initial adjuvant stimulation observed in deviation studies is of obvious significance to those in search of more efficient vaccines.

Proposed Course of Project:

Transfers involving lymphoid cell suspensions from other lymphoid organs and with T and B cell composition and of slices of lymphoid tissue from sensitized deviated donors will be made into normal or deviated recipients to assess role of cell-cell association or of certain tissue architecture in normal or deviated immune responses.

Honors and Awards:

Professorial lecturer in immunology, Howard University, Washington, D. C.
President, NIAID Assembly of Scientists

Publications:

Reiss, E., Stone, S. H., and Hasenclever, H. F.: Serologic and cellular immune activity of peptide-glucomannan fractions of Candida albicans cell walls. Infection and Immunity 9: 881-890, 1974.

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LABORATORY OF STREPTOCOCCAL DISEASES

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Summary Statement
Laboratory of Streptococcal Diseases
July 1, 1973 through June 30, 1974

Introduction

On June 11, 1973, the Laboratory of Streptococcal Diseases was established, and 14 personnel were transferred thereto on June 29, 1973, from the Laboratory of Microbiology being disestablished. This incorporates 5 projects previously listed under LM, and 2 new projects--reports of all of which are appended. In July 1974, Dr. Kenneth M. Nugent will complete 2 years as Research Associate, and will be succeeded by Dr. Harvey Edmonds. On July 1, 1973, Dr. Jon M. Ranhand was converted from Senior Staff Fellow and joined the staff as a Commissioned Officer, Sr. Scientist (T).

The mission of the Laboratory of Streptococcal Diseases is the advancement of knowledge of streptococci, and of other microorganisms, which may contribute to an understanding of genetic mechanisms, pathogenesis, and the relationships of structure and function of bacterial cell walls, membranes, organelles, biologically active molecules or aggregates, and bacteriophages.

Range of Ultrastructural Variations in Staphylococcus aureus Cell Wall Described

Studies of normal and mutant staphylococci by electron microscopy have enabled an initial synthesis of information of their ultrastructure, and particularly that of the cell wall. The approach has been to define "normal" wall, and to examine walls of known or suspected mutants in comparison with the normal. In thin sections, the variations that can occur are: (1) loss of trilaminar profile; (2) loss or decreased resolution of the submural region; (3) increased density of wall substance; (4) increased thickness of wall; (5) decreased density of wall; and (6) degrees of apparent lysis such as a widened wall profile, loss and degradation of wall substance, and undeniable lysis of cells. Obviously, some of these variations are opposites, and others occur in varying combinations. They are influenced by culture media and cell age, as well as by specified genetic defects leading to detectable biochemical deficiencies in some, but not all instances. The description of the possible variations establishes a reference for standardization of future correlative studies. (Cole, Popkin; Collaborators Chatterjee, Gilpin, Young, Tipper, not NIH)

Lysogeny Common in Group A Streptococci

The possibilities of alteration of streptococcal characteristics (antigens, toxins, etc.) by phage-mediated genetic exchange (transduction) or by the acquisition of prophage (lysogenic conversion) are being studied. A first requisite, however, is knowledge of the extent of lysogeny, because it can alter host

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susceptibility to phage adsorption or produce immunity to adsorbed related phages, as well as affect the characteristics directly. Reported studies, using indicator strains to detect phage produced from lysogenic cultures either spontaneously or by the action of inducers (ultraviolet or mitomycin C) place the extent of lysogeny among Group A streptococci at between 25 and 40%. Since indicator strains are frequently selectively specific, and it is unwieldy to use large numbers of such strains, we turned to direct electron microscopic examination (EM) of cultures exposed to mitomycin C. Among 44 randomly chosen strains, representing isolates made from 1950 to 1972 as well as some "classic" strains, the prevalence of lysogeny by EM was 100%. All the bacteriophages are morphologically identical, although differences can be shown by host range and serological studies. The importance of this high level of lysogeny must be determined by studies of prophage curing, as well as of transduction and lysogenization by heterologous phages, which are now in progress. (Colon, Cole, and Leonard).

Basic Characteristics of Temperate Bacteriophages of Group H Streptococci

Bacteriophages originating from Group H streptococci were discovered in this laboratory several years ago and have proven useful in genetic studies. Such utility is increased by cumulative knowledge of the phages and phage-host interactions, and investigations in these directions have been continuing. Using phage ø 227 grown in a Wicky strain, we previously determined methods for improved efficiency of phage recovery; the utility of a defined medium, latent and rise periods, burst size, and the non-dependence of absorption on divalent cations such as calcium. Current results show that production of vegetative (infectious) phage is inhibited by fluorodeoxyuridine, hydroxyphenylazouracil, rifampin, streptomycin, chloramphenicol, and incubation above 43 C; and decreased by nalidixic acid. In a rifampin-resistant host mutant, phage production is partially rifampin-resistant--apparently because the phage can utilize the mutant polymerase to which, however, rifampin binds and alters transcription efficiencies to some extent. Adsorption data suggest that the phage receptor is a polysaccharide. Biophysical studies show the phage molecular weight to be 42.29×10^6 daltons, its DNA content 35%, and therefore the DNA molecular weight to be about 15×10^6 daltons. The phage contains 6 structural proteins, which are coded for by 43.7% of the genome. Transfection studies with phage DNA showed dependence on presence of competence factor, on DNA concentration, on calcium and on the absence of competing species of DNA. In transfected cells, the lytic cycle is altered from that produced by infectious whole phage. Ongoing work emphasizes DNA conformation and molecular weight determinations by electron microscopy and by the agarose gel-ethidium bromide method. No such complete data are available in the literature for any streptococcal phage, and their availability will lead to new understanding of phage-host interactions in general, as well as to findings applicable to streptococcal problems. (Nugent)

Relation of Intracellular and Extracellular Hemolysins of Group A Streptococci Explores

The oxygen-stable or "surface" hemolysin of Group A streptococci, by which their presence on a blood-agar culture plate is usually suspected, is streptolysin (SLS). This extracellular SLS in active forms is bound to a ribonucleic acid (RNA) core, and prior studies of the present investigator showed that the streptococci contain an active cellular hemolysin (ACH) and a latent or potential cellular hemolysin (PCH)--both of which can be converted under appropriate conditions, to extracellular RNA-core SLS (EX-RNA-SLS). This year's concentration on PCH show that it is the probable usual precursor of EX-RNA-SLS, because, 1) PCH titers fall as EX-RNA-SLS titers rise during incubation in an appropriate medium; (2) two of 23 strains tested contained no PCH, and only these two failed to produce EX-RNA-SLS. An additional strain has been found, which produces PCH but not EX-RNA-SLS: and preliminary evidence suggests this is because of a defect in a metabolic activation step requiring energy or enzymes. The location of PCH appear to be in or on the cell membrane. New developments indicate that the identity of the other cellular hemolysin (i.e., ACH) and its separation from the known oxygen-labile hemolysin (SLO) also produced, will require further study. Future efforts will also focus on the toxic potential and renal binding of PCH as a possible factor in streptococcal-induced disease, as well as on genetic control of streptococcal hemolysins. (Calandra)

Muralysin Susceptibility of Aid in Defining "Group H" Streptococci

Strains of streptococci identified as S. sanguis are important in bacterial endocarditis, perhaps in dental caries, and as part of the normal oro-pharyngeal flora of man. They are also usually classified serologically as "Group H," although the group antigen is unidentified and the nature of presumed group-specific strains used for producing antisera, is in question. As part of studies to clarify this situation, we added a test of susceptibility to Group C streptococcal phage-associated lysin (PAL), which was used on 16 strains previously but tentatively identified as S. sanguis by other criteria. Eleven strains were lysed by PAL, although 3 were partially resistant. Extracts of the highly susceptible strains all reacted with an antiserum against one of the original Group H isolates, but not with another previously thought to be "Group H"; all possessed high levels of cell wall glucose and galactose; and all possessed all 5 antigens characterizing a "standard" S. sanguis strain. The 5 PAL-resistant strains did not possess these aggregate characteristics. Walls of PAL-susceptible strains were completely degraded by this muralysin, but neither they nor walls of PAL-resistant strains were sensitive to lysozyme unless treated with formamide: this, however, failed to render PAL-resistant walls susceptible to PAL. These findings suggest

that PAL sensitivity may be a useful adjunct screening procedure in presumptive placement of sanguis-like streptococci in Group H, as well as a rapid means of preparing protoplasts for examination of cellular fractions. Furthermore, the occurrence of different strains with walls reacting similarly to lysozyme, but differently to PAL, can provide data on both peptidoglycan structures and the undetermined site of action of PAL. (Calandra, Nugent, Cole, and Huff)

Effective Binding of Site of Competence Factor in Dispute

Published studies report that soluble competence factor (CF) in both pneumococci and streptococci, binds primarily to cytoplasmic membrane. It was concluded, therefore, that membrane is the site at which CF induces competence for genetic transformation. However, these findings were made on cells already fully competent, and do not answer the questions of the minimal amount of CF required for induction nor whether its initial effective location might not be obscured by excess and ineffective binding of CF elsewhere and later. Our studies show that pre-competent cells, exposed for 5 minutes to CF in the absence of protein synthesis, bind 0.01% of the maximal possible amount of bound CF--which is adequate for competence induction if the washed cells are now allowed to synthesize protein for 40 minutes without further exposure to CF. This trace amount of bound CF is found only in the cell wall portion of fractionated cells, and is trypsin-sensitive at this stage. We do not know if its location and trypsin-sensitivity may shift after initiation of protein synthesis--but this seems likely because a larger amount (1% of maximum), bound during a 5 minute exposure of pre-competent cells to CF during protein synthesis, is found primarily in the cytoplasmic membrane fraction and is now trypsin-insensitive. However, measurable CF levels are also present in wall and mesosome fracitons. After 40 minutes of exposure to CF during protein synthesis, as is routinely used for achieving full competence, maximum CF (100%) is bound in a trypsin-insensitive state and is distributed primarily and equally in membrane and mesosomes (although smaller amounts are present in wall and soluble fractions). Obviously, all these latter are in excess of the trace amount of CF actually required to induce competence and the shifts in location with changing conditions and time make it clear that the precise site for CF action has not yet been found. (Leonard, Colon, Cole, and Theodore)

Interdependence of Two New Proteins in Genetic Competence of Streptococci Shown

Earlier studies showed that streptococci, when capable of genetic DNA-mediated transformation (i.E., competent cells) contain at least 2 proteins absent from non-competent cells. These new proteins are manifest as an autolysin, and as a distinct band in electrophoresed polyacrylamide gels of cell extracts. New studies now show that the autolysin activity, which requires a reducing

agent with free sulfhydryl groups, is inhibited, as expected by sulfhydryl blocking agents such as mercuric chloride or N-ethylmaleimide. Simultaneously, competence achievement is prevented, although this was shown not to be due to inactivation of competence factor, inactivation of genetic markers, inhibition of cell growth or viability, inhibition of uptake of competence factor, nor prevention of uptake and expression of DNA by cells already competent. Both sulfhydryl-blocking agents were shown to inhibit de novo protein synthesis, including that of the new protein band. The complex data indicate that this new band and the autolysin are not the same protein detected in different ways, and that both are required for competence development. Adjunct experiments with inhibition of transformation after periodate treatment of competent cells at 37 C (oxidized carbohydrates and some amino acids in peptides) or at 0 C (oxidized only carbohydrates) suggest that the DNA binding site of competent cells is a protein attached to a carbohydrate core: the protein may be the new one shown on gels, but further study is needed. The cumulative data support the hypothesis that the autolysin dissolves a limited region of the cell wall and thereby exposes the other protein for binding of DNA. (Ranhand)

Serial No. NIAID 65
1. Streptococcal Diseases
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Electron microscopy of bacteria in relating structure and function

Previous Serial Number: Same

Principal Investigator: Dr. Roger M. Cole

Other Investigators: Mr. Terry J. Popkin
Dr. J. G. Tully, LID (Serial No. NIAID 207)

Cooperating Units: University of Arizona, College of Liberal Arts, Dept. of Microbiology and Medical Technology, Tucson (Dr. Neil H. Mendelson, Dr. John N. Reeve)

University of Rochester, School of Medicine and Dentistry, Dept. of Microbiology (Dr. Anadi S. Chatterjee, Dr. Frank E. Young)

University of Massachusetts Medical School, Dept. of Microbiology, Worcester (Dr. Donald J. Tipper, Dr. Carl M. Good)

University of California, San Diego, Dept. of Biology (Dr. W. Claiborne Brown)

Medical College of Pennsylvania, Philadelphia, Dept. of Microbiology (Dr. R. W. Gilpin)

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	18/12	-	-	18/12
Professional	6/12	-	-	6/12
Other	12/12	-	-	12/12

Project Description:

Objectives:

to examine the ultrastructure of microorganisms, in self-generated and collaborative studies, with particular emphasis on bacterial cell walls and membranes, their related antigens, and aspects of their participation in growth, division, genetic interchange, bacteriophage absorption, and the like.

Methods Employed:

Standard or newly-developed methods of bacteriology, serology, phage virology, microbial genetics, biochemistry, and electron microscopy. When appropriate, special methods of antibody labelling, cytochemistry, or other techniques of marking for electron microscopy.

Major Findings:

(A) Bacillus subtilis mutants

1. Collaborative studies of mutants defective in regulation of division, have continued (see previous reports: 1971-72; 1972-73). The tag-1 allele, responsible for temperature-sensitive phenotypic change in cell wall structure, was placed into an isogenic cell background together with a mutation (div. IV-B1) causing formation of minicells. Such minicells were purified by ultrasound procedures, which destroy parent cells. Although the usual tag-1-induced ultrastructural changes are not seen in walls of these minicells at any temperature, electron microscopy revealed that they autolyse more readily at 45 C than minicells without this mutation. The significance of this apparently different expression of the tag-1 mutation in minicells, as opposed to its usual 45 C expression in parental cells, is unclear, and further studies on ultrastructure and physiology of anucleate minicells are in progress. (Collaborators Mendelson and Reeve)

2. In 1971-72 (this project number) we examined ultrastructurally the progress of cell wall lysis by native autolysins at 3 different pH's (6.0, 8.0, and 9.5) in an asporogenous protease-negative mutant (strain SR22) of B. subtilis. No differences were found. In order to clarify the role of substrate (i.e., cell wall) in relation to the suspected 3 different autolysins (each active at a different pH), the phenomenon was reexamined by adding crude autolysin-containing fluid to cells which had been boiled in sodium dodecyl sulfate to inactivate native or wall-bound autolysins. These were incubated at pH 8.0 and timed samples (in ultrathin section) examined by electron microscopy. As with

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native autolysins, attack on wall progressed from cross-wall to peripheral wall to poles, and eventually resulted in complete wall destruction. These results, obtained without possible interference or enhancement by wall-bound lytic enzymes, suggest that the autolysins have no absolute specificity for any cell wall region, but that all attack wall in the same temporal sequence. (Collaborator: Brown)

(B) Staphylococcus aureus mutants

Studies of the last 2 years resulted in a synthesis of our current knowledge of ultrastructure of staphylococci, and particularly of their cell walls, which was presented at an international symposium of the New York Academy of Sciences (see publications) The attempt has been to define "normal" wall, and to view walls of known or suspected mutants under various conditions, against this standard. As seen by electron microscopy of thin sections, the variations which can occur are: (1) loss of trilaminar profile; (2) loss or decreased resolution of the submural region; (3) increased density of wall; (4) increased thickness of wall; (5) decreased density of wall; and (6) apparent degrees of lysis such as widened wall profile, loss and degradation of wall substance and cell lysis. These may occur in varying combinations, but our present knowledge does not permit prediction of their precise causes nor of their exact relationships to known biochemical and genetic defects. (Collaborators: Chatterjee, Gilpin, Young, Tipper)

(C) Spiroplasma

In efforts to determine the ultrastructure of the membrane, and particularly of the stellate discs which constitute, when stacked, striated bodies on the surface of this unique mycoplasma, membranes were prepared from large volumes of culture by osmotic shocking or by KCl treatment. The striated bodies or their subunits are released to some extent from cells or membranes by pronase treatment, but occur irregularly and in insufficient numbers for physical separation and purification. Pronase also induces appearance in or on membrane of 4.4 nm diameter long filaments which often appear associated or contiguous with packed arrays (sometimes hexagonal) of 7-8 nm rings; and of randomly-scattered larger (12-16 nm) rings. The stellate discs are seven-pointed (as determined by Markham rotation) and of the same diameter (12 nm) as the width of the striated bodies which they compose. The nature, function, possible origin from membrane components, and relation to similar but not identical structures in other mycoplasmas, of these bodies is being explored. (Collaborator: Tully)

(D) Electron Microscopy in support of other projects

1. Monitoring of phage (øFW227) production and developmental cycle in Group H streptococci, and purification of phage. (Project No. NIAID 65A-2; Nugent)

2. Determination of extent of lysogeny in Group A streptococci by electron microscopy after culture exposure to mitomycin C for induction of phage. (Project No. NIAID 65A; Colon)

3. Monitoring of sucrose gradient separation of membranes and mesosomes from competent Group H streptococci (strain Wicky), in studies of competence factor localization. (Project No. NIAID 65C; Leonard)

4. Verification of occurrence and extent of muralysin (phage-associated lysin; lysozyme) activity on cells and cell walls of Group H streptococci. (Project No. NIAID 65A-4; Calandra)

5. Monitoring of homogeneity of membrane and mesosome fractions of Staphylococcus aureus and a variety of other Gram-positive bacteria, in studies of localization of lipoteichoic acid. (Project Nos. NIAID 62 and 63B; Huff and Theodore)

6. Examination for ultrastructural changes, especially in mesosomes, occurring during penicillinase induction in Staphylococcus aureus 80/81: no differences from uninduced, penicillinase-negative cells were found. (Project No. NIAID 63B; Nugent and Theodore)

7. Examination, by negative staining and metal shadowing, of sarkosyl "M-bands" for verification of DNA-membrane complexes of Staphylococcus aureus isolated by this technique. (Project No. NIAID 63B; Twible, Theodore, and Nugent)

8. Investigations of the application of a low viscosity embedding medium (Spurr's) to bacteria and plant cells infected with mycoplasma. The results are as good, and in some respects better, than with embedments usually employed for section of bacteria. (Popkin)

9. Extensive monitoring of effects of breakage of bacteria by a new method (Vortexing-glass beads), and especially of production or liberation of new structures from enterobacteria. (New Project with Ranhand)

Significance to Bio-Medical Research and the Program of the Institute:

Definition of ultrastructure is a powerful and essential tool in the correlation of structure anf function. Our studies attempt

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to apply this to the understanding of cell envelope constituents (wall and membrane) in bacteria, by making use of normal (wild-type) and mutant strains of microorganisms which are cell-wall-bounded ("classic" bacteria) or which are only membrane-bounded (mycoplasmas, or occasionally induced bacterial L-forms). Our findings are beginning to provide a basis for ultrastructural recognition of genetic and biochemical effects on cell wall and on the cell division process; and for localizing essential growth and division functions in membrane and membranous organelles.

Proposed Course of Project:

1. Continuing examination of appropriate growth and division mutants of Gram-positive bacterial.

2. Continuing attempts to define the ultrastructure of Spiroplasma and related mycoplasmas-especially as regards membrane or cell envelope, and the cycle of bacteriophage.

3. Initiation of studies for ultrastructural localization of lipoteichoic acid in intact cells of Gram-positive bacteria.

4. Investigation of cell envelope and membrane structure in Gram-negative enterobacteria, as examined during sequential application of methods known to remove defined components; and of the related problem (as suggested by some preliminary observations) of regular aggregation or self-assembly of some liberated components.

5. Continued monitoring of streptococci, their components, and their bacteriophages, in support of genetic and other studies on these bacteria.

Honors and Awards:

Appointed to Editorial Board, Antimicrobial Agents and Chemotherapy, December 1973.

Continuing member, Editorial Board, Infection and Immunity.

Chairman-elect, Ultrastructure Division, American Society for Microbiology.

Elected to membership, Infectious Disease Society of America, 1972.

Project Officer, NIAID Research Contract PH 43-68-83, Univ. of Chicago.

Publications:

Cole, R. M., Tully, J. G., Popkin, T. J., and Bove, J. M.: Morphology, ultrastructure, and bacteriophage infection of the helical mycoplasma-like organism (Spiroplasma citri gen. nov., sp. nov.) cultured from "Stubborn" disease of citrus. J. Bacteriol. 115: 367-386, 1974.

Reeve, J. N., Mendelson, N. H., Coyne, S. I., Halloch, L. L., and Cole, R. M.: Minicells of Bacillus subtilis. J. Bacteriol. 114: 860-873, 1973.

Cole, R. M., Tully, J. G., Popkin, T. J., and Bove, J. M.: Ultrastructure of the agent of citrus "Stubborn" diseases. Ann. N. Y. Acad. Sci. 225: 471-493, 1973.

Feinman, S. B., Prescott, B., and Cole, R. M.: Serological reactions of glycolipids from streptococcal L-forms. Infection and Immunity 8: 752-756, 1973.

Mendelson, N. H., Reeve, J. N., and Cole, R. M.: Physiological studies of Bacillus subtilis minicells. J. Bacteriol. 117: 1312-1319, 1974.

Cole, R. M., Chatterjee, A. N., Gilpin, R. W., and Young, F.E.: Ultrastructure of teichoic acid-deficient and other mutants of staphylococci. Ann. N. Y. Acad. Sci. (In press)

Calandra, G. B., Nugent, K. M., and Cole, R. M.: Lysis of cells, degradation of walls, and formation of protoplasts of "Group H" streptococci by Group C streptococcal phage-associated lysin. J. Bacteriol. (In press)

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Serial No. NIAID 65A
1. Streptococcal Diseases
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on the Genetics of Streptococci

Previous Serial Number: Same

Principal Investigator: Alba E. Colon

Other Investigators: Carmen G. Leonard
Roger M. Cole

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other Personnel Assigned to Project	Guest Workers	TOTAL
Total	21/12	-	-	21/12
Professional	12/12	-	-	12/12
Other	12/12	-	-	12/12

Project Description:

Objectives:

To develop and apply systems of genetic exchanges, namely transformation, transduction and transfection, in streptococci. To study the effect of lysogeny on virulence and other properties of group A streptococci. To develop methods to detect and select transductants to various bacterial characteristics such as toxins, hemolysins, enzymes, and cell wall antigens.

Methods Employed:

The usual methods in microbial genetics, bacteriophage isolation and identification were employed.

Major Findings:

1. Transduction of streptomycin resistance in group H.

Last year we reported transduction by phage FW 227 of 4 nitrosoguanidine-induced mutants of strain SBEI/II. Now, following extensive testing for other transducible strains, we have found that only strain Challis-6, and one NTG

mutant of strain Wicky (strain M 8), were similarly transducible. However, whereas the SBE strain was transduced at equivalent efficiencies (ca $5x10^{-6}$) by either ultraviolet (UV)-irradiated or non-irradiated phage, Challis-6 and Wicky M8 were transduced only by UV-irradiated phage. Non-transducible parent strains and their transducible mutants adsorbed phage FW 227 equally well, and their competent cultures were transformed by streptococcal DNA and transfected by phage DNA, at equal efficiencies: the reasons for differences in transducibility are at present unknown and being explored. The genetic exchange in the transduced strains was shown to be phage-mediated by its failure in the presence of homologous phage anti-serum, by failure of desoxyribonuclease to inhibit it, and by its occurrence in non-competent cultures.

2. Transduction of serum opacity factor (SOF) in group A streptococci.

Some serotypes of group A streptococci-almost entirely those with difficulty-extractable and poorly-antigenic M protein-contain or produce a factor called serum opacity factor (probably a lipoproteinase) because such strains produce opacity in serum broths or around colonies on serum-agar plates. This factor may prove to be of importance in future efforts to purify proteins for human immunizations; and we felt it important to determine its genetic control. Since only phage-mediated transduction is operative in genetic interchange in group A streptococci, the initial problem was to propagate transducing phages in SOF^+ strains were exposed to transducing phages A25 and AT 298. In two strains, the phages propagated to high titer (10^9 PFU/ml); and one, made streptomycin-resistant, served as a donor, through the transducing phage, of the Str^R marker. Having thus shown the feasibility of transduction, the next problem is to determine feasibility of detecting and scoring possible transductants to SOF^+ on serum agar plates. At present, this is being explored by using quantitated mixtures of SOF^+ and SOF^- streptococci.

3. Studies in lysogeny.

There is a possibility that, as in some other bacteria, the presence of prophage (the lysogenic state) in group A streptococci can determine some important antigenic or other characteristics of the strain (lysogenic conversion). This has not been shown, nor, in fact, tested. A first approach is determination of the extent of lysogeny. Prior reports utilizing one to several indicator strains, and with and without use of an inducer such as mitomycin C, average about 40% lysogeny in group A. We avoided the use of indicator strains, which may be selectively specific, by direct electron microscopic examination of 44 randomly selected strains after mitomycin C induction. By this method all strains (100%) were lysogenic for at least one phage; all phages found were morphologically identical and therefore indistinguishable by this criterion. Although this higher-than-suspected incidence of lysogeny makes future work more difficult, it does not rule out lysogenic

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conversion-because many phages can be shown to be different by host range and serological studies. Another possible approach is to attempt to "cure" lysogeny (i.e., eliminate the prophage), and look for less of strain characteristics. To this goal, considerable time and effort was expended, in 1973-74 in growing quantities of given phages in order to produce specific antisera to be used in "curing" and in detecting lysogeny by more than one prophage per strain.

Significance to Biomedical Research and the Program of the Institute:

The genetics of streptococci remains relatively unexplored. Many advances, including all of those involved with phage-mediated genetic interchange, were initiated in this laboratory. These early studies were necessarily concerned with discovery, propagation, and characterization of appropriate phages-including those which transduce. Current and future studies are in the application of this knowledge to elucidation of the role of genetic exchange and lysogeny in nature-particularly in how such mechanisms may affect important attributes, such as antigenic characteristics and virulence or pathogenicity factors, among human pathogenic streptococci of groups A and H.

Proposed Course of Project:

We anticipate continuation of efforts to define the extent and importance of genetic transduction and lysogeny among streptococci. Although the group H system (which can also incorporate DNA-mediated transformational analysis) will continue to be useful as a model and for study of general phenomena related to microbial genetics, the most effort will concentrate on group A streptococci. The greatest need here is for development of methods for selection of transductants to streptococcal characteristics other than antibiotic resistance; and to determine suitable characteristics for study. Accordingly, we plan (1) to continue study of serum opacity factors; (2) to similarly examine control of hemolysin production (streptolysin S) in such SLS^+ and SLS^- strains as strain C203S and C203U; and (3) to initiate appropriate detection methods to determine if cell wall antigens are genetically or lysogenically convertible.

Honors and Awards:

None

Publications:

None

Serial No. NIAID 65A-2
1. Streptococcal Diseases
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Investigation of Group H streptoccal temperate bacteriophages

Previous Serial Number: Same

Principal Investigator: Dr. Kenneth M. Nugent

Other Investigators: Dr. Roger M. Cole

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	8/12	-	-	8/12
Professional	8/12	-	-	8/12
Other	-	-	-	-

Project Description:

Objectives:

To determine the fundamental characteristics of Group H streptococcal bacteriophage, including phage metabolic inhibitors), mechanism of phage adsorption, biophysical characterization (size, structural proteins, DNA content), and further characterization of transfection.

Methods Employed:

Routine bacteriological and bacteriophage techniques. Routine chemical analysis. Polyacrylamide gel electrophoresis. Sucrose gradient analysis.

Major Findings:

Phage metabolism. Production of vegetative (infectious) phage FW 227 is inhibited by fluorodeoxuridine (10 µg/ml),

hydroxyphenylazouracil (.2 mM0, rifampin (1 µg/ml), streptomycin (100 µg/ml) and chloramphenicol (100 µg/ml). Burst size is reduced 20% by nalidixic acid (100 µg/ml). Growth is also temperature-sensitive and incubation above 43 C stops pahge production. This inhibition is partially reversible by down-shifts to 37 C. The critical heat-labile step is not known.

Because production of ø FW 227 becomes rifampin resistant in a rifampin-resistant host, the phage must (be able to) utilize the B subunit of the host core polymerase-since it is this subunit that is functional under these conditions. However, the burst size and frequency of lysogeny are reduced in rifampin-resistant hosts, indicating that the mutation in the core polymerase causes a change in the transcription efficiency with phage DNA. If increasing concentrations of rifampin are added to rifampin-resistant bacteria during phage infeci-on, there is a progressive decrease in burst size. This suggests that although rifampin does not inhibit the mutant polymerase, it does bind to it and further changes the transcription efficiencies. (the presence of rifampin in a resistant bacterial culture does not change the growth rate nor the net rate of RNA synthesis of the bacterium.) Streptomycin sensitivity is relieved by growth of a streptomycin-resistant host. This indicates that the phage also utilizes host machinery at the translational level of protein synthesis.

Phage Adsorption. ø FW 227 adsorbs to stationary phase cells, heat-killed cells, trypsin-digested cells, and to cells in buffer without divalent cations. Citrate (100 mM) and EDTA (10 mM) slow the rate of adsorption, and of those phage which do adsorb, only 10% form infectious centers. Purified cell wall inactivates the phage. The receptor site is inactivated (extracted) by hot formamide, hot acid, and hot TCA, but in unaffected by 37 C alkali. These data suggest that a polysaccharide is necessary for adsorption.

Biophysical characterization. The molecular weight of ø FW 227 was calculated (by sucrose gradient sedimentation using lambda phage as an internal standard) to be 42.29×10^6 daltons. Analysis of phage purified by cesium chloride equilibrium centrifugation established that the phage is 35% DNA. These two determinations indicate that the molecular weight of phage DNA is 14.9×10^6 daltons. The purified phage was electrophoreses on neutral SDS polyacrylamide gels. The results showed six structural proteins (3 major, 3 minor) with an aggregate molecular weight of 434,300. Simple calculations (assuming a random unweighted amino acid composition) indicate that 43.7% of the phage genome codes for structural proteins.

Transfection. Purified ø FW 227 DNA is incorporated by competent WE 4 with the formation of infectious centers. This

uptake is absolutely dependent on the presence of CF and is stimulated by increasing amounts of CF. The formation of infectious centers is proportional to the DNA concentration squared, and is influenced by the ionic composition of the media (1 mM $CaCl_2$ stimulates, 1-10 mM $MgCl_2$ inhibits, 5 mM EDTA inhibits). The uptake of phage DNA is inhibited by bacterial DNA (either from the host strain or E. coli B) and this inhibition is proportional to the amount of carrier DNA. The lytic cycle resulting from the uptake of phage DNA in competent cells is significantly altered from the normal cycle induced by infectious whole phage. In the former, the latent period is extended from a normal of 45 minutes, to 65 minutes after DNA exposure; and the rise period is gradual from 65 to 88 minutes, instead of the normal 45-50 minutes. The burst size, however, is normal. In contrast, the single-step growth curve with whole phage and competent bacteria is identical to the normal vegetative cycle.

Significance to Bio-Medical Research and the Program of the Institute:

This work further characterizes Group H streptococcal temperate bacteriophage. This information should facilitate the use of these phages in investigating physiology and genetics of a group of important streptococci which has been little studied. Moreover, preliminary observations suggest that regulatory mechanisms in these phage-infected streptococci are complex and further investigation could provide fundamental insights into this type of biological problems in a streptococcal system which may differ in important respects from other bacterial systems.

Proposed Course of Project:

Since the principal investigator is separating from active duty in July 1974, there are no current plans to continue this specific work; but is anticipated that the fundamental findings will be applied to ongoing and future projects dealing with streptococcal genetics.

Honors and Awards:

None

Publications:

None

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Serial No. NIAID 65A-3
1. Streptococcal Diseases
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Investigation of the streptolysin S-related cellular hemolysins of Group A streptococci

Previous Serial Number: None

Principal Investigator: Dr. Gary Calandra

Other Investigators: None

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	9/12	-	-	9/12
Professional	9/12	-	-	9/12
Other	-	-	-	-

Project Description:

Objectives:

To determine the temporal and structural relationships a labile active cellular hemolysin and a latent or potential cellular hemolysin, both of which are functionally related to RNA-core streptolysin S (RNA-core SLS), and extracellular RNA-core SLS. Also, to determine if the cellular potential hemolysin is a factor in pathogenesis and could be an etiological factor in the development of poststreptococcal glomerulonephritis.

Methods Employed:

Routine bacteriological and biochemical techniques were employed.

Major Findings:

It had previously been discovered by the principal investigator that Group A streptococci contain both an active cellular

hemolysin (ACH) and a latent or potential cellular hemolysin (PCH) that can be activated by sonication or grinding. Both hemolysins can be converted to an hemolysin apparently identical to that of extracellular RNA-core SLS.

Maximal cellular titers of PCH are produced during late-log-phase growth in heart infusion - 0.3% maltose media. Cells harvested during this period and suspended in defined media with RNA-core (that fraction of Baker's yeast resistant to pancreatic ribonuclease) produce RNA-core SLS. During this time, cellular levels of PCH were found to fall almost to zero, whereas only a relatively small decline in PCH titers occurred in control cells incubated without RNA-core. This, in addition to previous studies, is evidence to support the hypothesis that extracellular RNA-core SLS is formed from PCH. If cells are incubated without RNA-core, they retain their potential to form RNA-core SLS only if incubated with maltose and cysteine, suggesting that an enzymatic or energy-requiring step may be necessary for activation of the potential hemolysin to RNA-core SLS. Additional support for this hypothesis is that one strain of Group A streptococci has been found which contains PCH, but does not form RNA-core SLS. This strain could have a defect in the postulated activation step.

The cellular location(s) of PCH has been determined after successfully preparing protoplasts of strain C203S. When suspended in 30% raffinose with 0.1 M Mg^{2+} (as previously reported by Dr. A. Schade) stable protoplasts can be prepared with the Group C streptococcal phage-associated lysin. Analysis of the various cellular fractions has shown that PCH is distributed as follows: membrane, 44%; cytoplasm, 34%; mesosome, 12%; and periplasm, 9%. PCH in the cytoplasm can be sedimented by high speed (200,000 x g x 2 hr) centrifugation, which suggests that most, if not all, of the hemolysin may be associated with a particulate fraction of the cell. PCH is not inactivated nor released from the membrane fraction by DNase or RNase, suggesting that the particulate fraction may be the cell membrane or membrane fragments.

The cellular titers of PCH were determined in 23 strains of Group A streptococci to determine whether it is unique to strain C203S or is found in others--including pathogenic strains. Of the strains tested, only two contained no potential hemolysin. One, C203U, is a mutant of C203S, and the other a type 33 isolated from a patient with bacteremia; neither strain forms RNA-core SLS. All strains isolated from patients with rheumatic fever (6) and glomerulonephritis (2) or nephritis (1) contain PCH. Of interest was the observation that some of these strains were non- to minimally hemolytic on blood plates under certain conditions. Removal of serum from the blood prior to preparing blood plates

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allowed these strains to produce unequivocal patterns of beta hemolysis.

Investigation of the active cellular hemolysin (ACH) has been difficult because of its marked lability, its low titer, and the presence of high titer streptolysin O (SLO) in the cellular lysate. N-ethylmaleimide, which inactivates sulfhydryl groups, has been used to inactivate cellular SLO, a sulfhydryl-dependent toxin. The residual hemolysin can be stabilized by treatment with streptomycin sulfate and dilution in 0.03 M sodium phasphate buffer, pH 6.5. The majority of this hemolysin is located in the periplasm. Although this hemolysin has properties of SLS such as stabilization by RNA-core, it has many of the characteristics of SLO--including a similar relationship of production to cell growth, kinetics of hemolysis of rabbit erythrocytes, and adsorption by sheep erythrocyte membranes. The identity of this hemolysin is uncertain at present and will require further investigation. As part of this study, the production, localization, and characterization of cellular SLO has been undertaken. Very high titer cellular SLO can be obtained under conditions developed for isolating PCH.

Significance to Bio-Medical Research and the Program of the Institute:

These studies have shown that the cellular SLS-related hemolysins are very different from the extracellular form. Since all studies on the pathogenic importance of the streptococcal hemolysins have been done on extracellular hemolysins, the perhaps truly important *in vivo* streptococcal membrane toxins have been ignored. The present studies have shown that potential hemolysin is produced by almost all of the pathogenic Group A streptococci thus far tested. Furthermore, many of the characteristics of the potential hemolysin are those of a toxin which could localize at the renal glomerular membrane and be a factor in the hemoglobinuria and hematuria in glomerulonephritis.

Proposed Course of Project:

1. Further procedures to purify PCH will be investigated. The possible pathogenic properties of potential hemolysin will then be tested on cells or cell cultures, and in test animals.

2. Attempts to determine the structure of the hemolytic moiety will be undertaken. If the amino acid composition can be determined and the hemolytic moiety pulse labeled, the prosynthesis of cellular SLS can be further studied.

3. Continued investigation of the properties and characteristics of the active cellular hemolysin will be done.

4. Related studies will include investigations into the biosynthesis of cellular SLO and to determine the characteristics of a presumed maltase of streptococci.

Honors and Awards:

Invited seminar, "Cellular SLS-related hemolysins of Group A Streptococci," Dept. of Medicine, Division of Infectious Disease, University of Texas Medical Center at San Antonio, May 1974.

Publications:

None

Serial No. NIAID 65A-4
1. Streptococcal Diseases
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Characteristics of "Group H" streptococci

Previous Serial Number: None

Principal Investigator: Dr. Gary Calandra

Other Investigators: Dr. Kenneth M. Nugent
Dr. Roger M. Cole
Dr. Eskin Huff

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	11/12	-	-	11/12
Professional	11/12	-	-	11/12
Other	-	-	-	-

Project Description:

Objectives:

To investigate various characteristics (biochemical reactions, morphology, phage-host ranges, antigenic structure, cell wall structure, etc.) of those streptococci, usually designated as Streptococcus sanguis or "Group H", which may aid in defining the group. Specifically, in this year, to determine their susceptibility to lysis by a cell wall lysin (Group C streptococcal phage-associated lysin, or PAL).

Methods Employed:

Routine techniques of bacteriology, biochemistry, and phage virology, and electron microscopy.

Major Findings:

PAL lyses streptococci of Group A, which are insusceptible to lysozyme unless specially pre-treated. To determine this relationship in strains lumped as "Group H", as well as to test feasibility of preparing protoplasts by treatment with wall-lytic enzymes, we tested 16 strains in our collection. Whole cells of 8 strains were lysed by PAL; 3 strains were relatively resistant and 5 were completely unsusceptible. None were sensitive to lysozyme, nor were their isolated walls. However, the isolated walls of susceptible strains (but not of others) were completely degraded by PAL, as determined by electron microscopy. All PAL-susceptible strains reacted with a "Group H" antiserum (vs. strain Blackburn), but not with antiserum to a strain (K208) once erroneously labelled as "Group H": the PAL-insusceptible strains reacted oppositely. Moreover, PAL-susceptible strains usually possessed all 5 antigens of a standard Streptococcus sanguis (strain M5), whereas insusceptible strains possessed only a few or none of these antigens.

In attempts to clarify the nature of PAL action, as related to cell wall differences, and to response to a wall-lytic enzyme (lysozyme) of known site of attack, walls of susceptible and insusceptible strains were treated with various chemical procedures before exposure to either enzyme. Acid extraction was ineffective in all instances. Formamide extraction, the effects of which were enhanced by subsequent de-O-acylation, rendered all walls susceptible to lysozyme--whether such walls came from PAL-susceptible strains: it did not simultaneously alter the non-suceptibility of PAL-insusceptible walls. These results indicate that aspects of wall structure which are known to influence lysozyme activity, are not operative for PAL.

Protoplasts of strain WE4 were prepared, using PAL with 30% raffinose and 0.1 M mg^{2+} added to the incubation mixture. Although PAL was most active in the presence of EDTA and was partially inhibited by Mg^{2+}, protoplasts remained stable only in the presence of the high Mg^{2+} concentration. DNA, subsequently liberated from the protoplasts, was biologically active in that it could mediate transformation of rifampin resistance. Further cellular studies have not yet been undertaken.

Significance to Bio-Medical Research and the Program of the Institute:

In addition to their intrinsic biological and genetic interest, streptococci considered to be Streptococcus sanguis or "Group H" are under increasing study as agents of endocarditis and oral-dental lesions. Their inclusion in a single serologic group is in some dispute, and additional characterization is needed. The

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studies described suggest that cell wall structure, as determined by screening for susceptibility to lysis by Group C streptococcal phage-associated lysin (PAL), may prove to be a useful adjunct criterion for clarifying taxonomic position of sanguis-like streptococci. In the laboratory, susceptible and unsusceptible strains provide a new means for determing the site of PAL action, and consequently for elucidating the chemical structure of the cell walls. Similarly, the demonstrated capacity to prepare protoplasts is expected to facilitate studies of cellular products such as competence factor or competence factor or competence-associated autolysin, which are currently being examined in other projects of this Laboratory.

Proposed Course of Project:

As time and personnel permit, we expect to continue characterization of increasing numbers of streptococcal strains which appear to be Str. sanguis or "Group H". The characterizations will include morphology and ultrastructure, colony type, hemolysis, biochemical reactions, phage-host ranges, serology and antigenic structure--as well as the most immediate problem of determining what factors, or what aspects of cell wall structure and chemistry, are related to the resistance or susceptibility of streptococcal walls to PAL or other muralytic enzymes.

Honors and Awards:

None

Publications:

Calandra, G. B., Nugent, K. M., and Cole, R. M.: Lysis of cells, degradation of walls, and formation of protoplasts of Group H streptococci by Group C streptococcal phage-associated lysin. J. Bacteriol. (In press)

Serial No. NIAID 65C
1. Streptococcal Diseases
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Reports
July 1, 1973 through June 30, 1974

Project Title: Competence development and genetic transformation in Group H and Group A streptococci

Previous Serial Number: Same

Principal Investigator: Mrs. Carmen G. Leonard

Other Investigators: Miss Alba E. Colon
Dr. Roger M. Cole
Dr. Theodore S. Theodore

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	33/12	-	-	33/12
Professional	12/12	-	-	12/12
Other	23/12	-	-	23/12

Project Description:

Objectives:

To define and understand the events induced by competence factor(s) (CF), which initiates the genetically competent state in Group H streptococci. Current efforts are directed to localization and identification in the bacterial cell of the sites for CF binding and subsequent uptake of DNA

Methods Employed:

The usual methods in microbial genetics were employed. In addition, streptococcal cell fractions were prepared by protoplasting (with Group C streptococcal phage-associated lysin) in hypertonic raffinose-containing buffer (method of Schade, NIAID 63C, 1971-72)--followed by differential centrifugations, protoplast lysis, etc. to separate them into cell walls, cytoplasmic membranes,

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mesosomal vesicles, and soluble cytoplasmic material (See Theodore et al, NIAID 63B, 1971-72, for methods). Some fractions were prepared by direct mechanical cell breakage, omitting the protoplasting step. All fractions were monitored for homogeneity by electron microscopy. CF activity in each of the cell fractions was determined by releasing the bound CF by heating the fraction at pH 2.0, and by subsequent competence-producing assay of the released material.

Major Findings:

We reported earlier that only added or extracellular CF that is adsorbed by pre-competent cells is the effective inducer of competence in either CF producing (Challis) or CF-non-producing (Wicky) cultures. We also reported the requirement for different periods of protein synthesis for competence development after CF addition.

Studies reported here were initiated in efforts to localize the binding sites of CF in pre-competent cells and to follow any possibly different attachment sites of CF throughout the period of competence induction and subsequent DNA uptake.

To accomplish this we have done extensive fractionation of streptococcal cells (Wicky) after different periods of exposure to added CF. These experiments have also been performed with and without added chloramphenicol to separate the different known period of protein synthesis required for competence development. In these studies we have used culture supernatant fluids of strain Challis as a source of free CF. Since it is known that optimal competence induction depends on the concentrations of both cells and added CF, it was first necessary to determine the concentration of these CF preparations required for saturation of about 1×10^8 colony forming units/ml of strain Wicky (50 min old). It was found that optimal transformation frequencies of around 5% were obtained when 5% (v/v) CF was used. It was also found that these cells adsorbed from the cultures supernatants over 95% of the added CF during the 40 min incubation period required for full competence development.

Conditions and procedures were developed to recover and quantitate cell-bound CF from the isolated fractions. Total cell-bound CF is defined as the amount of CF extracted from the washed, intact cells, prior to their fractionation. Total cell-bound CF is determined for each fractionation experiment and arbitrarily represents 100% value of bound CF. The amount of CF in each cell fraction is then determined and is expressed as a percent of the total cell-bound CF.

Using modifications of the fractionation procedures described, we obtained concentrated mesosomal and membrane fractions from

Group H streptococcus, strain Wicky, of high homogeneity (as observed by electron microscopy).

By mechanical disintegration, we obtained clean cell-wall fractions from strain Wicky cells.

Using these conditions and procedures, we made the following observations:

1. A 5 min contact between added CF and pre-competent Wicky cells (50 min old), in the absence of protein synthesis, resulted in sufficient binding of CF to lead to competence if subsequent protein synthesis (about 40 min) was allowed, but otherwise the cells were not and did not become competent. This cell-bound CF was sensitive to inactivation by trypsin. This work confirmed previous results, and additionally verified the initial binding by direct measurements of the amount (as activity) of CF bound during the first 5 min contact. The amount of CF bound was less than 0.01% of the amount that these cells are normally capable of binding during competence development. This trace amount was found in the cell wall fraction and no detectable level of CF was found in the other fractions.

2. When similar pre-competent cells were exposed to CF for 5 min during concomitant protein synthesis, over 1% of the total amount of CF that these cells are capable of binding, was bound. After this 5 min period of protein synthesis the cells were not competent but the cell-bound CF is no longer sensitive to inactivation by trypsin. In addition, the bulk of the cell-bound CF was found in the membrane fraction, although measurable trace amounts were also found in cell wall and mesosome fractions.

3. When similar cells were exposed to CF for 40 min during concomitant protein synthesis (as required for full competence development), most of the added CF was absorbed from the supernatant fluids and large amounts appeared as cell-bound CF. (This amount of cell-bound CF was used as the 100%, or maximal, standard). These cells were fully competent and this bound CF was no longer sensitive to trypsin inactivation. In fractions of these fully competent cells this bound CF was found predominantly in, and equally divided between, mesosomes and cytoplasmic membrane; but lower activities were also measured in the cell wall and soluble fractions.

It can then be concluded that only trace amounts of cell-bound CF are actually required for competence induction, although pre-competent Wicky cells can bind over 1,000-fold more CF than needed. However, since measurable amounts of cell-bound CF were found at different and multiple sites (cell wall, membrane,

mesosome, and soluble fractions) after the onset of protein synthesis, it is impossible at present to determine where the competence-inducing effect of CF is actually initiated.

These results raise significant doubts of published concepts (based on both pneumococcal and streptococcal data) that since the bulk of the cell-bound CF in already competent cells is found in the membrane fraction, the membrane must be the binding and induction site for CF action. In those published studies the low levels of CF found in other fractions were not considered significant, nor were timed studies involving pre-competent cells included. Obviously, further work along these lines is required, in order to determine the induction site and mechanism of action of this extremely active biological product.

Significance to Bio-Medical Research and the Program of the Institute:

The present findings that only trace amounts of CF are required for competence induction, although significant excess amounts may be bound by the pre-competent cells, further emphasizes the possible hormone-like action of CF. The findings further emphasized the need for new approaches and more quantitative techniques to determine how such small quantities of this biologically active product can induce such permeability changes in a bacterial cell which then allows extracellular DNA to be taken in and thus be transformed. Obviously, an understanding of this mechanism would be of great value in other systems involving DNA uptake.

Proposed Course of Project:

We plan to continue studying the mechanism of competence induction in Group H streptococci. We want to determine the nature of CF and of the CF and DNA binding sites in the bacterial cell. We plan to study further the effects of lysogeny in transformation.

Honors and Awards:

Received Superior Service Award, May 1974.

Publications:

Leonard, C. G.: Early events in development of streptococcal competence. J. Bacteriol. 114: 1198-1205, 1973.

Serial No. NIAID 65C-1
1. Streptococcal Diseases
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Competence development and genetic transformation in Streptococcus sanguis: nature and role of autolysins.

Previous Serial Number: Same

Principal Investigator: Dr. Jon M. Ranhand

Other Investigators: None

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	24/12	-	-	24/12
Professional	12/12	-	-	12/12
Other	12/12	-	-	12/12

Project Description:

Objectives:

To define the role that autolysins play in the development of competence in Streptococcus sanguis cultures.

Methods Employed:

Standard microbiological and chemical methods are used. See previous reports for details.

Major Findings:

Evidence presented in an earlier report showed that competence development (the ability of some bacterial strains to react with exogenously supplied deoxyribonucleic acid, DNA, and thereby become genetically changed or transformed) involved the induction

of new proteins. One protein was manifested as a single band in polyacrylamide gels after subjecting acidified-phenol extracts of whole competent cells to electrophoresis. The other protein was manifested as an autolysin (an enzyme capable of causing limited dissolution of the bacterial cell wall producing cell lysis under certain conditions).

Recent kinetic evidence suggested that the development of competence was integrated with the expression of the autolytic enzyme; both the cells' ability to autolyze and their ability to undergo genetic transformation occurred simultaneously. Moreover, in 17 strains of Streptococcus sanguis studied, cells that could transform but could not autolyze, were not found. The converse, however, was found. These latter cells possibly do not undergo genetic transformation for other reasons, i.e., they lack the ability to bind DNA or to recombine DNA.

Since the role of autolysins in competence development was only correlative, I attempted to prove or disprove their direct involvement in the process. Earlier evidence suggested that the autolysin required a reducing agent (2-mercaptoethanol) for maximum activity. This observation inferred that a free sulfhydryl group was necessary for activity. Therefore, an attempt was made to inhibit competence development by specifically inhibiting the function of the autolysin with chemicals that react with sulfhydryl groups, i.e., mercuric chloride or N-ethylmaleimide. Neither reagent interfered with the uptake of competence factor by cells nor with the uptake and expression of DNA by competent cells. Neither reagent inactivated competence factor nor genetic markers coded by the transforming DNA, nor did they inhibit cell growth or viability appreciably. However, results obtained with either N-ethylmaleimide or mercuric chloride showed that the inhibition of competence development was related to both the inhibition of the autolytic enzyme activity and the inhibition of the biosynthesis of the other protein described above. It was also revealed for the first time that these two proteins are not one-and-the-same. The latter protein may be directly involved with binding DNA to cells. Moreover, the data obtained show that either chemical can selectively inhibit the de novo biosynthesis of proteins.

In other experiments, it was shown that the competent cells' ability to bind DNA was due to a moiety that contains amino acids, i.e., a protein or polypeptide. This was demonstrated directly by treating competent cells with sodium meta periodate at either 0 C or at 37 C. At 37 C, periodate oxidizes both carbohydrates and certain amino acids in peptide chains; at 0 C, periodate oxidizes only carbohydrates. When competent cells were treated with periodate (5 mM) at 37 C for 10 min, they lost

92% of their ability to undergo genetic transformation; at 0 C, they lost only 50%. The decrease observed at 37 C was directly related to a decrease in the cells ability to bind DNA. When 10 mM periodate was used, transformation decreased 96% at 0 C and 99% at 37 C. This result strongly suggests that the DNA binding proteins are attached to a carbohydrate backbone or core.

Significance to Bio-Medical Research and the Program of the Institute:

The nature of genetic competence in bacteria, which can be likened in some respects to an infectious process as regards the uptake of naked deoxyribonucleic acid (DNA), is unknown. Continuing findings from this project have indicated the importance of a cell-wall-splitting enzyme (autolysin), and another protein newly-formed in competent cells, in achieving DNA entry and binding in Streptococcus sanguis (Group H). This bacterial system, which can be turned on by a defined, soluble competence factor and for which (from associated projects) we have adjunct information on bacteriophages and bacteriophage-mediated genetic exchange, offers the best opportunity among transformable bacteria for delineating the nature and sequence of steps, which culminate in new genetic information in the recipient cell.

Proposed Course of Project:

Although the nature of the newly-synthesized competent cell-associated protein is beginning to be clarified, its location is still unknown. In addition, the nature of the autolysin--as determined by end products of cell wall dissolution by a purified preparation--has yet to be discovered. I plan to continue investigation in these directions.

Honors and Awards:

Lecturer, NIH Graduate School, Foundation for Advanced Education in the Sciences.

Participant at the XVII Annual Meeting on Microbial Transformation, Topoco, North Carolina; June, 1973.

Publications:

Ranhand, J. M.: Autolytic activity and its association with the development of competence in Group H streptococci. J. Bacteriol. 115: 607-614, 1973.

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Ranhand, J. M.: Inhibition of the development of competence in Streptococcus sanguis (Wicky) by reagents that interact with sulfhydryl groups. Discernment of the competence process. J. Bacteriol. (in press, June 1974)

Ranhand, J. M.: Inhibition of transformation by sodium metaperiodate in Streptococcus sanguis (Wicky). Demonstration that deoxyribonucleic acid binding sites contain amino acids. J. Bacteriol. (In press, June 1974)

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LABORATORY OF BIOLOGY OF VIRUSES

1974 Annual Report

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Laboratory of Biology of Viruses

Research efforts of the Laboratory of Biology of Viruses continue to be directed toward learning how human and other animal viruses infect cells, replicate, integrate into the host genome and produce the transformed state. Attention is given to the elucidation of specific viral functions which could provide targets for future viral chemotherapy. The Laboratory is distinguished by the molecular and biochemical approach used in these studies. The past year has been a highly productive one and many notable findings with regard to the structure, synthesis and transcription of the viral genetic material and to the characterization of unique viral enzymes and structural proteins have been made. Members of LBV form a cohesive unit of indivuduals who are expert in different areas of molecular biology. Dr. Norman P. Salzman, the laboratory chief has spent the past year in the Department of Molecular Biology at the University of Geneva learning advanced methods of electron microscopy while Dr. Bernard Moss has served as Acting Chief.

Structure, synthesis, and transcription of papovavirus DNA

A major portion of the laboratory efforts have been directed to the papovaviruses. This group of small DNA viruses is of particular interest because of their ability to transform cells and their recent isolation from patients with neurological diseases and immunological deficiencies. New studies on the structure, synthesis and transcription of the DNA of SV40 and other papovaviruses are summarized below.

Cleavage of polyoma DNA with restriction endonucleases. Restriction endonucleases have previously been used in this laboratory to specifically cleave SV40 DNA. These studies have now been extended to polyoma virus. Four fragments representing 61%, 26%, 8%, and 5% of the original DNA were obtained with the restriction enzyme R·Hin. These fragments were ordered by analysis of partial digest products (Chen and Salzman).

Localization of single-stranded regions in superhelical SV40 DNA. The single-stranded regions of superhelical SV40 DNA were labeled with a radioactive water soluble carbodiimide and analyzed with restriction endonucleases. The bound substituent was associated with 3 of the 11 cleavage fragments (Chen, Lebowitz, and Salzman, N.).

Presence of pyrimidine runs in SV40 DNA. Analysis of SV40 DNA indicates the presence of several long tracts of pyrimidines in one copy per SV40 genome. They would not be expected on a purely random basis and presumably are present for an important biological function (Kasselberg and Salzman, N.).

Mechanism of SV40 DNA replication. SV40 DNA chain growth occurs by a discontinuous mechanism in which 4S DNA fragments are made and then joined to growing DNA chains. Analysis of these fragments by polyacrylamide gel electrophoresis indicates considerable heterogeneity in size (Thoren and Salzman, N.).

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New method for isolation of a restriction enzyme from Hemophilus parainfluenza. The restriction enzyme Hpa I from Hemophilus parainfluenzae was isolated and characterized after purification by a new method. The enzyme was used to cleave SV40 DNA, mitochondrial DNA and Vero cell DNA into unique fragments (DeFilippes).

Defective forms of SV40 DNA. Propagation of SV40 at high input multiplicities has been shown to result in extensive alterations and rearrangements of viral genetic information. The reassortment of viral genetic information has resulted in the generation of circular DNA molecules consisting of the reiteration of a specific one-third of the viral genome. In all cases, the initiation site for DNA replication has been preserved. In the "triplicated" circular viral DNA we have studied most extensively, the sites of recombination which give rise to the rearranged genome, correspond very closely with those present in several of the adeno-SV40 hybrid viruses. Such sites may assume an important role during the integration of the SV40 genome into cellular DNA (Martin, Howley, Khoury).

Biochemical relationship of human isolates of papova viruses to SV40. The DNA of a papova virus isolate from the urine of patients receiving renal transplants (BK virus) and SV40 DNA have been compared. The studies indicated that BK DNA is circular, supercoiled, and slightly smaller than SV40 DNA. At present, the virus DNA examined is considerably more heterogeneous than SV40 which may reflect the methods used for its propagation. Nucleic acid hybridization studies suggest that 10-20% of the sequences present in BK DNA are homologous to SV40 DNA (Howley, Martin, Khoury).

Isolation of specific SV40 DNA sequences by affinity chromatography. A method of affinity chromatography based on RNA-DNA hybridization was developed for specific sequence isolation. SV40 DNA fragments were covalently immobilized to neutral cellulose powder by a water soluble carbodiimide. SV40 cRNA, synthesized *in vitro*, was securely immobilized onto phosphocellulose powder activated by carbonyldiimidazole. The immobilized SV40 DNA or RNA is able to efficiently isolate complementary SV40 RNA or DNA sequences in the presence of vast excess of unrelated sequences in a continuous hybridization system (Shih, Martin, Khoury).

Topographical location of SV40 DNA sequences expressed in virus-free transformed cells. The predominant class of stable, viral specific RNA is complementary to the "early" region of the SV40 genome. In addition, less abundant species of viral RNA are present in transformed cells which are transcribed from regions of SV40 DNA immediately adjacent to the "early" region. This latter class of viral-specific RNA is complementary to DNA sequences located predominantly at the 5' end of the "early" region and may reflect "read through" of the integrated SV40 genome from cellular DNA. In its integrated form (i.e., linked to cellular or other viral DNAs) the minus strand of SV40 DNA is preferentially transcribed into stable RNA species (Khoury and Martin).

Expression of SV40 DNA from Adeno-SV40 hybrid viruses. The non-defective Adeno-SV40 hybrid viruses ($AdND^{+}$) contain DNA consisting principally of

adenovirus DNA and varying, but specific portions of SV40 DNA. The expression of SV40 DNA "integrated" into such adenovirus DNA molecules was evaluated by reacting unlabeled RNA from cells productively infected with Ad ND^+ virions. In each case DNA sequences present on the "early" DNA strand reacted with infected cellular RNA; no hybridization was observed with the "late" strand of SV40 DNA. In addition the extent of the reaction was related to the amount of SV40 DNA present in the hybrid virus genome (Khoury and Martin).

Transcription of SV40 RNA from chromatin of transformed cells. SV40 specific RNA was transcribed from chromatin of SV40 transformed cells by E. coli RNA polymerase. It was complementary to the minus strand of SV40 DNA (Shih, Martin, and Khoury).

Papovavirus Proteins

The genome of papovaviruses is large enough to code for only 6 to 8 different proteins, thus making it feasible to eventually identify all the gene products.

Isolation of SV40 induced proteins from infected cells. Affinity chromatography employing immunosorbents prepared with antibody against SV40 virus specific proteins has been used as a probe for the isolation of early viral proteins. Approximately 5 to 10 percent of the polypeptides synthesized after infection are identical to the two major structural proteins of the virion, the coat proteins. These appear after the onset of viral DNA synthesis. Prior to viral DNA synthesis, there appears two polypeptides which are virus induced and not structural proteins. One of the proteins has a molecular weight of approximately 70,000 (Barban).

Subunit composition of the SV40 capsid proteins. Previous studies suggested that the main coat protein of SV40 is composed of two identical subunits. Additional evidence has now been obtained by amino acid analyses and peptide mapping technique to substantiate the earlier findings (Barban).

Isolation of a protein that binds to DNA. A protein which binds to denatured DNA and a specific 55S nucleoprotein complex has been isolated from SV40 infected cells (Sebring, Zweig, Salzman, N.).

Structure and Synthesis of Adenovirus DNA

The adenoviruses are human pathogens that also possess the ability to transform cells. Significant new information about the structure and synthesis of the DNA of these viruses, which is larger than that of the papovaviruses, is summarized below.

Arrangement of repeated sequences near the termini of adenovirus 18 DNA is characterized. The DNA of all adenoviruses so far examined contains an inverted terminal repetition. Owing to these repetitious sequences the ends of denatured DNA molecules can self-anneal to form single-stranded circles closed by a duplex hydrogen-bonded segment. However, in contrast to the circular molecules produced with DNA from all other serotypes, there

was associated with each Ad 18 circular molecule a visible, apparently duplex, projection representing approximately 6% of the genome. Occassionally longer duplex projections have also been observed. Based on apparent resistance to N. crassa nucleases, denaturation in 87% formamide, and detection of specific heteroduplexes predicted from annealing of strands which contain terminal repetitions of different lengths we conclude that Ad 18 DNA has an unusually large (occasionally variable) inverted terminal repetition and that single-stranded circles are closed by a duplex, antiparallel base-paired "panhandle" (Garon and Rose).

Cleavage of adenovirus heteroduplexes into unique fragments using N. Crassa nucleases. Heteroduplex molecules constructed with DNA from several adenovirus serotypes could be clearly separated from homoduplexes using a benzoylated-naphthoylated-DEAE-cellulose (BND) column. Treatment of the heteroduplex fraction with single-strand specific nucleases from N. crassa produced fragments which correspond in size to the three double-stranded regions of the intact heteroduplex molecule. The fragments, free of both internal single-strand breaks and single-strand tails have been isolated preparatively from neutral sucrose gradients. Renaturation kinetic analyses support the conclusion that each fragment represents a unique segment of the adenovirus genome. The treatment of purified heteroduplex molecules with single-strand specific nucleases provides a procedure for generating preparative quantitites of both physically and genetically defined fragments by a method that does not depend on the recognition of sequence-specific cleavage sites. Furthermore, the procedure is potentially applicable to the fractionation of any duplex polynucleotide molecule that contains uniquely located, and perhaps artificially introduced, distortions of the helix (Garon and Rose).

Analysis of single-stranded DNA synthesized during infection suggests mechanism of adenovirus DNA synthesis. Single-stranded fragments of adenovirus type 2 DNA can be isolated from infected KB cells under conditions which retarded reassociation of complementary sequences but do not denature native viral DNA. Of the total intracellular, virus-specific DNA, recovered from cells at high efficiencies after a 1 hr pulse with ^{3}H-dThd beginning 15 hr after infection, about 20% is single-stranded when fractionated on hydroxyapatite. This DNA shifts to the double-stranded fraction on hydroxyapatite during an extended chase incubation suggesting the presence of single-stranded DNA in replicating intermediates. Furthermore, single-stranded DNA anneals nearly equally to both strands of the adenovirus genome. These findings are consistent with a model of DNA replication involving strand-displacement with synthesis of the displaced strand being initiated from either end of the parent template (Garon and Rose).

Phleomycin, an antibiotic with antitumor activity, produces single-strand breaks in viral DNA. Phleomycin was found to produce single-strand breaks in purified adenovirus and phage deoxyribonucleic acid. A reducing agent was required and both native and single-stranded deoxyribonucleic acids were substrates for this activity. The limit reaction products were acid insoluble and sedimented at 5 S or less in sucrose density gradients. The deoxyribonucleic acid cleaving activity of phleomycin could be measured by a nitrocellulose filter binding assay and characteristics of this assay

are described. Ribonucleic acid did not appear to be cleaved by phleomycin (Garon and Rose).

Structure, synthesis and transcription of parvovirus DNA

The parvoviruses are DNA-containing viruses which are even smaller than the papovaviruses and have recently been implicated in the pathogenesis of viral hepatitis and viral gastroenteritis. Two members of this group, adenovirus-associated virus (AAV), a defective virus that only replicates in the presence of adenovirus and Kilham rat virus, a non-defective virus, have been investigated.

Messenger RNA transcribed from the AAV genome may represent a single, monocystronic species. Viral RNA analyses revealed two populations of AAV RNA, (i) a single, discrete 20S species and (ii) a very heterogeneous population ranging in size from 4S to 18S. In the nucleus both populations of AAV RNA were observed but in the cytoplasm the 20S RNA was mainly present in the polysome fraction and the heterogeneous RNA was mainly in the non-polysomal fraction. The 20S RNA, therefore, appears to be the only functional AAV message species. The heterogeneous AAV RNA may arise from incomplete transcription or degradation of the 20S RNA in the nucleus or cytoplasm. The molecular weight of the 20S AAV RNA was approximately 0.9 to 1.0 x 10^6 which accounts for the entire region of the AAV genome that is stably transcribed. This 20S RNA may be monocistronic and thus AAV DNA may contain only one gene (Carter and Rose).

Electron microscopic heteroduplex mapping technique used to analyze genetic relatedness among AAV serotypes. When heteroduplex DNA molecules were prepared with any combination of AAV-1, -2 or -3 DNA, regions containing heterology melted out progressively as denaturing conditions were increased. This indicates that partial homology is present within these regions, and suggests that sequence differences among the serotypes have arisen by retention of random base changes. In addition, interserotypic homologies physically mapped at similar locations, indicating that the same sequences are conserved in all three serotypes. No melting was observed in homoduplex molecules spread under similar conditions. Because single-stranded circles remain essentially intact under the same spreading conditions, open heteroduplex ends mean that the terminal self-complementary sequences are not completely conserved among different serotypes. Although competition hybridization indicates that at least 60-70% of sequences in AAV-1, -2 and -3 DNA are homologous, heteroduplex melting studies suggest that interserotypic homologies actually approach 90-95% (Rose and Koczot).

Heteroduplex structure indicates terminal base arrangement in AAV DNA. When heteroduplex DNA molecules, prepared with any combination of AAV-1, -2, or -3 DNA, were spread in high concentrations of formamide, self-annealed strand ends could be specifically located and "panhandle" structures readily identified. These projections confirmed the mode of terminal base arrangement which previously had been uncertain. From the length of these projections it was estimated that the terminal repetition in AAV DNA is equivalent to 4-8% of total strand DNA (100-200 base pairs) (Koczot and Rose).

DNA polymerase associated with purified KRV and the cell line in which it is grown were purified and characterized. A DNA polymerase associated with purified Kilham rat virus (KRV) has been solubilized and characterized. The enzyme requires 4 nucleoside triphosphates, a divalent cation either Mg^{++} or Mn^{++}. The enzyme activity in the presence of Mg^{++} is stimulated two to three fold by the addition of 0.02 M KCl. The enzyme has a strong template preference for a synthetic DNA polymer containing deoxyadenylate and deoxythymidylate.

The DNA polymerases from the rat nephroma cell line in which KRV is grown were also studied. Two polymerases were identified in the cell cytoplasm and two additional major ones in the nucleus. The enzymes were all purified, characterized and compared to the viral associated enzyme. One cytoplasmic enzyme C_{II} resembles the KRV-associated enzyme in its stimulation by 0.02 M KCl but differs from the viral associated enzyme in its template specificity and cation requirements (Salzman, L.).

Kilham Rat Virus specific RNA synthesized after infection. RNA from infected cells was shown to be specific for KRV by analysis of its nucleotide base ratios and hybridization with KRV and cellular DNA. Viral RNA synthesis was detected by 2 hours after infection of cells prior to viral DNA synthesis is initiated. The viral RNA has a sedimentation coefficient of approximately 18S in dimethylsulfoxide-sucrose gradients and a calculated molecular weight of 6.5 to 7.5 x 10^5. If the viral specific RNA is a homogeneous species, it would account for 40 to 50% of the viral genome (Salzman, L.).

Characterization and synthesis of poxvirus enzymes

Laboratory interest in the poxviruses, the largest and most complex of the DNA viruses, has been directed chiefly toward the enzymes contained within the core. These enzymes may be involved in transcription of the early genes.

Purification and characterization of a poly A polymerase from vaccinia virus. Eukaryotic and viral mRNA species contain long poly A sequences. A poly A polymerase was purified several hundred fold from vaccinia virus cores. The most important property of the enzyme is its ability to add adenylate residues to RNA or DNA primers. This was demonstrated by a variety of methods including changes in sedimentation of the poly A after ribonuclease or deoxyribonuclease digestion and by isotope transfer or nearest neighbor analysis (Moss).

Purification and characterization of a novel protein kinase from vaccinia virus. Protein kinases have been shown to play a role in regulation in eukaryotic cells. The incorporation of ^{32}P-ATP from γ ^{32}P-ATP into viral proteins by partially disrupted vaccinia virus is stimulated by protamine. A protein kinase that phosphorylates specific virus proteins and which is dependent on protamine for activity was purified several hundred fold from vaccinia cores and extensively characterized. In addition, 2 virus acceptor proteins were isolated and characterized by gel filtration, peptide mapping and amino acid analysis (Kleiman and Moss).

Induction of nucleic acid-dependent phosphohydrolases following vaccinia virus infection. Two nucleic acid-dependent phosphohydrolases were purified from vaccinia virus cores and shown to be immunologically distinct. Both enzymes were induced in HeLa cells following vaccinia infection; they could not be detected in uninfected cells. Further studies with metabolic inhibitors and specific antiviral drugs indicated that the enzymes are synthesized de-novo after vaccinia infection, primarily after the stage of viral DNA synthesis (Paoletti and Moss).

Stability of HeLa cell mRNA following vaccinia virus infection. The inhibition of host protein synthesis by vaccinia virus is accompanied by a rapid decrease in the size of polyribosomes. This polyribosome breakdown is not caused by extensive degradation of HeLa cell mRNA and other possible mechanisms are being explored (Rosemond-Hornbeak and Moss).

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Serial No. NIAID-80A
1. Biology of Viruses
2. Physical Biochemistry
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Biochemical Characterization of Nucleic Acids of Transformed and Virus Infected Cells

Previous Serial No.: Same

Principal Investigators: Dr. Malcolm Martin
Dr. George Khoury
Dr. Peter Howley
Ms. Janet Byrne
Mr. James Schmidt

Cooperating Units: Dr. Kenneth Takemoto
Dr. Stuart Aaronson
Dr. Daniel Nathans, Department of Microbiology, Johns Hopkins School of Medicine, Baltimore, Md.

Man Years:

	NIAID Lab. Staff	Other Personnel Assigned to Project	Guest Workers	Total
Total:	51/12			51/12
Professional:	30/12			30/12
Other:	21/12			21/12

Project Description:

Objectives:

To elucidate the process of viral DNA transcription under conditions of productive and transforming infection.

To examine the mechanisms involved in the integration of viral genetic material into the animal cell genome.

To determine the relationship between papovaviruses isolated from human sources and laboratory forms of these agents such as SV40 and polyoma.

Methods Employed:

Simian virus (SV) 40 was propagated in green monkey cells and purified

by isopycnic centrifugation in CsCl. SV40 DNA and cellular DNA were prepared by SDS treatment and phenol extraction from uninfected, productively infected or transformed mammalian cells. Viral DNA was analyzed by isopycnic centrifugation, velocity rate sedimentation under neutral and alkaline conditions, electrophoresis through agarose or polyacrylamide gels, sensitivity to specific bacterial restriction endonucleases and nucleic acid hybridization. SV40 specific RNA was prepared from lytically infected or transformed monkey cells and analyzed by hybridization techniques employing separated strands of viral DNA.

Major Findings:

A. SV40 DNA Transcription

1. In collaboration with Dr. Daniel Nathans we reported the topographical location of DNA sequences complementary to stable species of "early" and "late" lytic RNA on the physical map of the viral genome. These studies were extended to a series of virus-free transformed cells. In each case, the predominant species of viral RNA was complementary to DNA sequences physically located in the "early" region of the viral genome. In addition, a less abundant yet stable class of RNA was present in the transformed cell lines examined which specifically reacted with the portions of the "early" (or minus) DNA strand that were adjacent to the "early" region. This latter class of viral RNA does not appear in a stable form during the lytic cycle and is currently being evaluated in studies dealing with factors which regulate viral gene expression.

2. The non-defective Adeno-SV40 hybrid viruses ($AdND^+$) contain DNA consisting principally of adenovirus DNA and varying, but specific portions of SV40 DNA. The expression of SV40 DNA "integrated" into such adenovirus DNA molecules was evaluated by reacting unlabeled RNA from cells productively infected with Ad ND^+ virions. In each case DNA sequences present on the "early" DNA strand reacted with infected cellular RNA; no hybridization was observed with the "late" strand of SV40 DNA. In addition the extent of the reaction was related to the amount of SV40 DNA present in the hybrid virus genome.

B. Defective forms of SV40 DNA

Propagation of SV40 at high input multiplicities has been shown to result in extensive alterations and rearrangements of viral genetic information. We have examined and characterized two separate isolates of SV40 each of which is homogeneous and consists solely of reiterated SV40 DNA.

1. Serial passage of one isolate resulted in the appearance of viral DNA molecules containing 3 Eco_{RI} restriction endonuclease sites symmetrically located on the circular viral genome. Analyses of such molecules by nucleic acid hybridization studies as well as heteroduplex mapping monitored electronmicroscopically has indicated that two distinct portions of the SV40 genome, both located in the "late" region of the molecule,

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have recombined to form a DNA segment one-third the size of viral DNA. Three such segments have then become linked resulting in a circular DNA molecule approximately the same size as SV40 DNA. Such "triplicated" molecules contain three initiation and termination sites for DNA replication. In addition, the sites of recombination are located at regions of the SV40 DNA corresponding to the segments of the viral present in some of the Ad-SV40 hybridiviruses.

2. A second isolate, analyzed in a similar manner, appears to consist of DNA sequences that are topographically located in the "early" region of the viral genome which are triplicated to yield DNA circles which are slightly smaller than the parental SV40 DNA.

C. The Biochemical Relationship of human isolates of papova viruses to SV40.

In collaboration with Dr. Kenneth Takemoto, we have compared the DNA of a papova virus isolate from the urine of patients receiving renal transplants (BK virus) and SV40. Our results indicate that BK DNA is circular, supercoiled, and slightly smaller than SV40 DNA. At present, the virus DNA examined is considerably more heterogeneous than SV40 which may reflect the methods used for its propagation. Nucleic acid hybridization studies suggest that 10-20% of the sequences present in BK DNA are homologous to SV40 DNA.

Significance to Biomedical Research and the Program of the Institute:

The ultimate goal of this project is to understand the integration of viral genetic material into the mammalian chromosome and the relationship of this process to oncogenesis. The determination of the time course of integration, the number of copies of viral DNA present in cells undergoing transforming infection and the nature of the integration site in the animal cell genome may permit one to separate the various steps involved and possibly interrupt these molecular events with appropriate chemotherapeutic agents.

We are also interested in delineating those viral genes necessary to induce and/or maintain the transformed state. Utilizing the separated strands of SV40 DNA, we will be able to characterize those viral gene products that are present in tumor cells from those required for SV40 productive infection. A more thorough understanding of the control mechanisms which modulate viral DNA transcription may enable one to regulate the type of cellular response to these agents.

Proposed Course of the Project:

Emphasis will be placed on understanding the mechanisms of integration of both RNA and DNA tumor virus genetic material into the host chromosome(s). Mammalian DNA from cells undergoing both productive and transforming infection will be examined to determine when stable integration occurs. Studies are currently underway to biochemically evaluate papova viruses isolate from human sources.

Honors and Awards:

Malcolm Martin
Site visits at:
University of Mississippi
UCLA
Ad Hoc Member, Virology Study Section
Invited lectures at:

College of Physicians and Surgeons, Department of Biochemistry, University of Maryland
Columbia University, Department of Biology
ICN-UCLA Symposium on Molecular Biology
Conference on the Biology of Cancer, University of Michigan
NATO Conference on Tumor Virology, Monte Carlo
Perspectives in Biochemistry, Clinical Center, NIH.

Dr. George Khoury

Invited lectures at
McGill University, Montreal, Canada, April 27, 1973
New York University Medical School, May 15, 1973
University of Uppsala, Uppsala, Sweden, September 14, 1973
Karolinska Institute, Stockholm, Sweden, September 10, 1973
University of Alabama Medical Center, October 2, 1973
Imperial Cancer Research Foundation, London, September 15, 1973
University of Amsterdam, Amsterdam, Holland, September 19, 1973
NATO Conference, Monte Carlo, September 4, 1973
Harvard Medical School, May 15, 1973
Princeton University, October 18, 1973
University of Pennsylvania, January 24, 1974

Publications:

Khoury, G., Martin, M. A., Lee, T. N. H., Danna, K. J., and Nathans, D.: A map of SV40 transcription sites expressed in productively infected cells. J. Mol. Biol., 78:377-389, 1973.

Fareed, G. C., Khoury, G., and Salzman, N. P.: Self-annealing of 4S strands from replicating SV40 DNA. J. Mol. Biol., 77:457-462, 1973.

Martin, M. A., and Khoury, G.: Transcription of SV40 DNA in lytically infected and transformed cells. In Virus Research, 33-50. ed. Fox, C. F., and Robinson, W. S., Academic Press, New York and London, 1973.

Shih, T. Y., Khoury, G., and Martin, M. A.: In Vitro transcription of the viral-specific sequences present in the chromatin of cells transformed by SV40. Proc. Natl. Acad. Sci., U.S.A., 70:3506-3510, 1973.

Gelb, L. D., Milstien, J. B., Martin, M. A., and Aaronson, S. A.: Characterization of Murine leukemia virus-specific DNA present in Normal Mouse cells. Nature New Biol., 244:76-79, 1973.

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Martin, M. A., Gelb, L. D., Fareed, G. C., and Milstien, J. B.: Reassortment of simian virus 40 DNA during serial undiluted passage. J. Virol., 12:748-757, 1973.

Shih, T. Y. and Martin, M. A.: A general method of gene isolation. Proc. Nat. Acad. Sci., U.S.A., 70:1697-1700, 1973.

Khoury, G., Lewis, A. M., Oxman, M. N., and Levine, A. S.: Strand orientation of SV40 transcription in cells infected by non-defective adenovirus 2-SV40 hybrid viruses. Nature New Biol., 246:202-205, 1973.

Serial No. NIAID-80G
1. Biology of Viruses
2. Macromolecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Characterization and Biosynthesis of Viral Enzymes and Structural Proteins.

Previous Serial Number: Same

Principal Investigators: Dr. Bernard Moss
Dr. Enzo Paoletti
Dr. Hortencia Rosemond-Hornbeak
Dr. Jay Kleiman
Dr. Cha Mer Wei
Mr. Norman Cooper
Mr. Alan Gershowitz

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	60/12		16/12	76/12
Professional:	36/12		16/12	52/12
Other:	24/12			24/12

Project Description

Objectives:

1. Identification, isolation and characterization of viral enzymes and structural proteins with the aim of elucidating their roles in viral replication.

2. Determination of regulatory mechanisms involved in the biosynthesis of viral proteins.

3. Analysis of post-translational modifications of viral proteins and their relationship to enzyme function and virion assembly.

4. Demonstration of the effects of antiviral drugs on the function and biosynthesis of viral proteins and on the assembly of virions.

Methods Employed:

Vaccinia virus was grown in HeLa cells and purified by density gradient sedimentation. Biosynthesis of macromolecules was determined by incorporation of radioactive precursors and separation by gel electrophoresis, gel filtration, column chromatography or rate zonal and equilibrium centrifugation. Enzymes were purified by column chromatography and were assayed by measuring the transformation of radioactively labeled precursor molecules. Structural analysis of purified macromolecules was made by enzymatic and chemical hydrolysis followed by electrophoretic and chromatographic analysis.

Major Findings:

1. Purification and characterization of a polyriboadenylate polymerase. Eukaryotic and viral messenger RNA species contain a sequence of 100 to 200 adenylate residues at the 3' terminus. Little is known regarding either the function of this polyriboadenylate sequence or the enzymes involved in their synthesis. Last year we described the isolation of a poly A polymerase from purified vaccinia virions. We have now purified the enzyme extensively by a combination of DEAE cellulose and DNA cellulose chromatography and characterized it. The most important property of the poly A polymerase is its ability to add adenylate residues to synthetic RNA or DNA primers. This was demonstrated by a variety of methods including changes in sedimentation of the poly A after ribonuclease or deoxyribonuclease digestion and most rigorously by isotope transfer experiments. Transfer of ^{32}P from α ^{32}P-ATP to 2'(3')-CMP was obtained after alkaline or enzymatic digestion of the poly C primed reaction product and transfer to 3'-dTMP was found after enzymatic hydrolysis of the poly dA:dT primed product.

2. Purification and characterization of a protein kinase. Enzymes which phosphorylate proteins are involved in the regulation of cell metabolism. Recently protein kinases have been found associated with several different animal viruses. Vaccinia virions catalyze the incorporation of ^{32}P from $\gamma^{32}P$-ATP into viral P-acceptor proteins. Incorporation into these viral proteins, which have been identified by autoradiography following polyacrylamide gel electrophoresis (PAGE), is stimulated at least five fold by the addition of protamine, but unaffected by cyclic nucleotides. The protein kinase (PK) responsible for this phosphorylation has been purified approximately 200 fold from vaccinia cores. P-acceptor proteins were resolved into two purified components of molecular weight 38,000 (CM I) and 12,000 (CM II) daltons. Tryptic peptide mapping demonstrated that ^{32}P labeled CM I and CM II contain different phosphorylated peptides; ^{32}P-phosphoserine and phosphothreonine were identified by electrophoresis of acid hydrolysates. The purified PK system requires three protein components for activity: viral enzyme, CM I or CM II, and protamine. PAGE analysis of the reaction products showed that over 97% of the total ^{32}P incorporated was in CM I or CM II; label in protamine accounted for less than 3%. Protamine seems to act as an activator or reaction intermediate to regulate the in-vitro vaccinia PK reaction. Such a mechanism has not been previously described, and is reminiscent of cyclic-AMP mediated control of protein phosphorylation. A protamine-like endogenous viral protein could control in-vivo protein phosphorylation.

3. Induction of nucleic acid-dependent nucleoside triphosphate phosphohydrolases during vaccinia virus replication. Two phosphohydrolases (I) and (II) which hydrolyze ATP to ADP and Pi have been purified from vaccinia virus cores. The activities of both enzymes were greatly stimulated by the addition of nucleic acids but they differed in substrate specificities and polynucleotide cofactor requirements. Antisera prepared against the individual enzymes neutralized their activities and indicated that they were immunologically distinct. Examination of extracts from uninfected and vaccinia infected HeLa cells showed that phosphohydrolase activity increased after vaccinia infection. Enzymes corresponding to I and II were obtained by chromatography of extracts of infected cells on DNA-cellulose columns. The induced enzymes were identified by their substrate specificities and were neutralized with specific antisera. Similar enzymes were not detected in uninfected HeLa cells; the cellular phosphohydrolase activities were not adsorbed to DNA-cellulose, stimulated by nucleic acids or neutralized. Further experiments indicated that the induced enzymes appeared primarily after the stage of viral DNA synthesis and that actinomycin D or cycloheximide prevented this. Rifampicin, a drug which blocks vaccinia morphogenesis did not inhibit enzyme induction. Thus, phosphohydrolases I and II are immunologically distinct enzymes that are synthesized de novo after vaccinia infection.

4. Stability of HeLa cell mRNA following vaccinia virus infection. The inhibition of host protein synthesis which follows vaccinia virus infection is accompanied by a rapid decrease in size of polyribosomes. This polyribosome breakdown occurs even when actinomycin D is used to prevent the synthesis of virus RNA. The possibility that polyribosome breakdown results from degradation of the host messenger RNA was investigated. HeLa cell mRNA labeled with ^{3}H-uridine was isolated by chromatography on poly U agarose columns. Virus infection did not alter either the amount or the size, as estimated by sucrose gradient sedimentation, of the HeLa cell mRNA. Thus the breakdown of polyribosomes is not due to extensive degradation of host RNA.

Significance to Biomedical Research and the Program of the Institute:

Enzymes have been found associated with both RNA and DNA-containing virions but very few have been isolated and characterized. The solubilization of the vaccinia virus core and the purification of 5 separate enzymes represents a significant advance in this field. We should now be able to fully characterize the enzymes and possibly learn their precise role in virus replication. In addition these viral enzymes have unique properties which distinguish them from previously described cellular enzymes and thus are potential targets of anti-viral compounds.

Proposed Course of the Project:

During the next year we expect to further characterize the 5 isolated virion enzymes and to study their biosynthesis and assembly into virions. It is anticipated that the drug rifampicin will be a unique tool for the latter studies. We also plan to look for additional enzymes that might be involved in the processing of RNA.

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Honors and Awards:

Bernard Moss - Editorial Board of Journal of Virology
Editorial Board of Antimicrobial Agents and Chemotherapy
Editorial Board of Intervirology
Invited lecture: Gordon Research Conference on Cells and Viruses

Hortencia Rosemond-Hornbeak - Invited lectures at:
Vanderbilt University School of Medicine
University of South Alabama Medical School

Enzo Paoletti - Invited lectures at:
Gordon Research Conference on Animal Cells and Viruses
Columbia University, College of Physicians and Surgeons
Purdue University, Dept. of Biological Sciences
New York State Dept. of Health, Division of Laboratories and Research, Infectious Disease Center
The M. S. Hershey Medical Center, The Pennsylvania State Univ.
The Wistar Institute
Georgetown University
Roswell Park Memorial Institute
Mt. Sinai School of Medicine
University of Connecticut

Publications:

Rosemond, H. and Moss, B.: Phosphoprotein component of vaccinia virions. J. Virol. 11: 961-970, 1973.

Moss, B., Rosenblum, E. N., and Garon, C. F.: Glycoprotein synthesis in cells infected with vaccinia virus, III. Purification and biosynthesis of the virion glycoprotein. Virology 55: 143-156, 1973.

Kleiman, J. and Moss, B.: Protein kinase activity from vaccinia virions: Solubilization and separation into heat-labile and heat-stable components. J. Virol. 12: 684-689, 1973.

Moss, B.: Rifamycin SV derivatives. In Carter, W. A. (Ed.): Selective Inhibitors of Viral Functions. CRC Press, Cleveland, 1973, pp. 313-328.

Moss, B., Rosenblum, E. N., and Paoletti, E.: Polyadenylate polymerase from vaccinia virions. Nature 254: 59-63, 1973.

Moss, B. and Rosenblum, E. N.: Protein cleavage and poxvirus morphogenesis: tryptic peptide analysis of core precursors accumulated by blocking assembly with rifampicin. J. Mol. Biol. 81: 267-269, 1973.

Paoletti, E., Rosemond-Hornbeak, H., and Moss, B.: Two nucleic acid-dependent nucleoside triphosphate phosphohydrolases from vaccinia virus: purification and characterization. J. Biol. Chem., in press.

Paoletti, E. and Moss, B.: Two nucleic acid-dependent nucleoside triphosphate phosphohydrolases from vaccinia virus: nucleotide substrate and polynucleotide cofactor specificities. J. Biol. Chem., in press.

Rosemond-Hornbeak, H., Paoletti, E., and Moss, B.: Single-stranded DNA specific nuclease from vaccinia virus: purification and characterization. J. Biol. Chem., in press.

Rosemond-Hornbeak, H. and Moss, B.: Single-stranded DNA specific nuclease from vaccinia virus. Endonucleolytic and exonucleolytic activities. J. Biol. Chem., in press.

Moss, B.: Reproduction of poxviruses. In Fraenkel-Conrat, H. and Wagner, R. R. (Ed.): Comprehensive Virology, Vol. I. Plenum Press, New York, 1974, in press.

Moss, B. and Rosenblum, E. N.: Vaccinia virus polyriboadenylate polymerase: covalent linkage of the product with polyribonucleotide and polydeoxyribonucleotide primers. J. Virol., in press.

Serial No. NIAID-80H
1. Biology of Viruses
2. Molecular Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on the Replication of the Parvovirus, KRV

Principal Investigator: Dr. Lois A. Salzman

Other Investigators: Ms. Louese McKerlie

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	24/12			24/12
Professional:	12/12			12/12
Other:	12/12			12/12

Project Description:

Objectives:

1. To solubilize, purify and characterize the DNA polymerase found associated with the KRV virion.

2. To compare the viral-associated DNA polymerase with the DNA polymerases from the rat nephroma cell used for growth of KRV.

3. To study the synthesis of KRV specific ribonucleic acid in regard to the time of synthesis after virus infection of a cell and the size of the viral RNA.

Methods Employed:

KRV was extracted from the cells in which the virus grew and purified by at least two isopycnic centrifugations in cesium chloride. The purified virions had DNA polymerase activity. Brief sonication of the virions in the presence of dithiothreitol and the non-ionic detergent NP-40 followed by incubation with desoxycholate resulted in a solubilization of the enzyme. The enzyme was then separated from contaminating proteins by chromatography on DEAE cellulose, DNA cellulose and phosphocellulose columns. The purified enzyme was characterized

according to its pH optimum, cation requirement substrate and primer-template specificity, molecular weight and other associated enzyme activities. The enzyme assay involves the incorporation of the nucleotide ^{3}H-deoxyadenosine triphosphate into acid insoluble material.

The rat nephroma cell line in which the virus was grown was also examined for its content of DNA polymerases. The cells were broken by gentle shear in a Dounce homogenizer. The extract was separated into nuclear and cytoplasmic fractions. The nuclear and cytoplasmic fractions were separately examined for DNA polymerase activity. The enzymes were purified from contaminating proteins by the same chromatographic steps as the viral associated polymerase using DEAE-cellulose, DNA cellulose and phosphocellulose columns. The enzymes were then characterized as described above for the viral associated enzyme.

Studies on specific viral ribonucleic acid (RNA) synthesis involved the incorporation of the radioisotope ^{3}H-uridine into the RNA of infected cells. The radioisotope was added at various times after KRV infection of the cells. The RNA was extracted from the cells and shown to be virus specific by analysis of its nucleotide base ratio and by hybridization with viral and cellular DNA.

Major Findings:

KRV-associated DNA polymerase. The DNA polymerase associated with purified KRV has been solubilized and purified from contaminating proteins. The enzyme is found in a single peak of activity after chromatography on DEAE-cellulose, DNA-cellulose and phosphocellulose columns. The KRV-associated DNA polymerase has increased enzyme activity in the presence of 0.02 M KCl and has a strong preference for a synthetic DNA polymer containing deoxyadenylate and deoxythymidylate. The enzyme appears to be a single protein band on polyacrylamide gels. It has an approximate molecular weight of 73,000 $\pm$ 5,000 and appears to contain endonuclease activity.

Rat nephroma cell DNA polymerases. Using DEAE cellulose chromatography two separable DNA polymerase enzymes were found in the cytoplasmic fraction and two major DNA polymerases were detected in the nuclear fraction of the cell. One nuclear enzyme can be separated on DNA cellulose into 1 major and two minor components. The enzymes were all purified, characterized and distinguished from each other by several criteria. The activity of cellular cytoplasmic enzyme C_{II}, is stimulated by the addition of KCl and in this respect resenbles the viral associated enzyme. The viral associated and the cytoplasmic enzyme C_{II} differ however, in template specificity and cation utilization.

Synthesis of KRV-specific RNA. A portion of the RNA (0.5 to 1.0%) synthesized after infection of a permissive cell with the virus KRV is virus specific. The specificity was demonstrated by analysis of its nucleotide base ratios and by hybridization with KRV and cellular DNA. Viral RNA is synthesized as early as two hours after infection before viral DNA synthesis occurs. The viral RNA has a sedimentation coefficient of approximately 18S in dimethylsulfoxide-sucrose gradients and a calculated molecular weight of 6.5 to 7.5 x 10^{5}. If the viral RNA is a homogeneous species, it would account for 40 to 50% of the viral genome.

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Significance to Bio-medical Research and Program of the Institute:

Kilham rat virus (KRV) first isolated from a rat sarcoma is a member of the parvovirus group. Members of the group have been found in many mammals and birds as well as lepidoptera and bacteria. H1 virus closely related to KRV and the defective virus AAV are human parvoviruses. The human gastroenteritis and hepatitis B viruses are also believed to be parvoviruses. The parvoviruses are very small (18 to 22 nm) and those studied contain one molecule of linear single-stranded DNA with a molecular weight of 1.5 to 2.2 x 10^6. The viral DNA is capable of coding for very few proteins. With this small amount of information, the virus replicates and causes cell disintegration. Little is known about the method of virus control of the mammalian cell and virus replication. Using KRV as a model it may be possible to determine some of the important aspects of the virus cell interaction.

None of the steps involved in the synthesis of linear single-stranded virion DNA are understood. There may be a double-stranded DNA intermediate involved and from this intermediate the single-stranded virion DNA may be made. We hope that further studies with the purified virus-associated DNA polymerase may aid in an understanding of the DNA replication of parvoviruses.

The parvoviruses are also of interest because of their interaction with oncogenic viruses. KRV has been reported to suppress induction of leukemia in rats by the RNA maloney virus. (Bergs, Cancer Res. 29: 1669, 1969). H1 can reduce the incidence of spontaneous and induced tumors in hamsters. The adeno-associated virus (AAV) inhibits transformation of hamster embryo cells and induction of tumors in vivo by the oncogenic adenovirus 12. An understanding of the replicative cycle of the parvovirus may aid in the understanding of this suppression of some cancer manifestations.

KRV and H1 are capable of causing latent or inapparent infections. The presence of latent viruses in cells, their effect on the cell and the cause of activation of these latent viruses are of considerable interest to those studying malignancy and other disease states. The parvoviruses because of their apparent simplicity may serve as excellent models for the study of latent diseases.

Proposed Course of the Project:

1. We hope to more closely define the role, if any, of the purified viral associated DNA polymerase in virus replication. Very little is known about the replication of linear single-stranded DNA and studies with this enzyme may aid in clarifying the steps involved.

2. Investigation of the viral RNA synthesized by the infected cell will be continued. We hope to use the synchronized cell cultures and isolate the RNA from the polysomes for hybridization and sedimentation studies.

3. We hope to study further the synthesis of viral DNA in an infected cell. We will look specifically for any intermediates involved in the viral replication as determined by sedimentation in sucrose gradients, column chromatography and observation in the electron microscope.

4. We also wish to determine the extent of cellular DNA inhibition and the time when such inhibition occurs. This information may aid in gaining insight into how the virus gains control of the infected cell.

Honors and Awards:

Invited participant at a Symposium on Parvoviruses in February, 1974.

Publications:

1. Salzman, L. A. and Redler, B.: Synthesis of viral specific RNA in cells infected with the parvovirus, KRV. J. Virol. 14: in press.

2. Salzman, L. A.: Properties of the Kilham rat virus associated DNA polymerase. J. Infec Dis. 130: in press.

Serial No. NIAID-84C
1. Biology of Viruses
2. Biochemical Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Mechanism of DNA Replication and Integration

Previous Serial No.: Same

Principal Investigators: Dr. Norman P. Salzman
Dr. Edwin D. Sebring
Dr. Alfred G. Kasselberg
Dr. Michael Chen
Dr. Martin Zweig
Ms. Marilyn Thoren

Other Investigators: None

Cooperating Units:

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	60/12	--	12/12	72/12
Professional:	48/12	--	12/12	60/12
Other:	12/12	--	--	12/12

Project Description:

Objectives:

To further define the mechanism of SV40 DNA replication with emphasis on the process of DNA chain elongation and termination.

To correlate biologic function of oncogenic DNA viruses with the structure of their DNA's.

To identify and characterize the viral and cellular specified proteins involved in the replication of SV40 DNA and maturation of virus particles.

Methods Employed:

Tissue culture cells are infected with SV40 virus and at appropriate times after infection radioactive precursors are added to the medium. The replicating molecules of SV40 virus and/or SV40-specific nucleoprotein complexes are then fractionated from the cellular material and further purified and characterized by isopycnic centrifugation and velocity sedimentation. The replicating molecules are denatured to release the newly synthesized strands which are then subjected to polyacrylamide gel electrophoresis. Other methods used in this laboratory have included: fractionation of the SV40 genome with various restriction endonucleases followed by gel electrophoresis; base ratio determinations using ion-exchange chromatography; production of pyrimidine isostichs by acid digestion of SV40 DNA and separation of the isostichs by DEAE cellulose chromatography.

Major Findings:

Previous studies in this laboratory have established that SV40 replication is initiated at a specific site on the viral genome. Replication proceeds bidirectionally with 4S DNA fragments being synthesized and later joined to a growing DNA chain. Gel electrophoresis should provide a more accurate size measurement of this 4S fragment. By comparison with molecules of known molecular weight, in the same size range, it should be possible to determine whether these fragments are immediately joined to the growing chain or whether there is some lag period. Considerable difficulty has been encountered in establishing gel conditions which will permit the sizing of these very small single-stranded DNA fragments. Preliminary results indicate that the "4S" fragments are a very heterogeneous group (M. Thoren, N. Salzman).

In another study, SV40 DNA uniformly labeled with ^{32}P was subjected to digestion with formic acid and diphenylamine. This treatment releases purine bases and breaks the phosphodiester backbone of DNA adjacent to the purine bases. Therefore, the products of such a digestion are inorganic phosphate, free purines, and runs of various lengths containing only pyrimidine nucleotides. A quantitation of the pyrimidine runs present in the SV40 genome was carried out. There are several long runs which are present in one copy per SV40 genome. In addition, there are several shorther pyrimidine runs which are present in large excess of the number expected from a random distribution of bases in the genome. Localization of pyrimidine runs in the SV40 genome and correlation of their presence with biologic functions are being carried out. (A. G. Kasselberg and N. Salzman).

An analysis of the superhelix density of SV40 replicating DNA molecules has indicated the presence of a denaturated region, presumably at the replicating forks. A protein which binds to denaturated DNA has been isolated from SV40 infected cells by chromatography on a denatured DNA-cellulose column. This protein is currently being characterized to determine if it may be associated with the denatured regions of replicating DNA molecules.

In other studies, an examination of nucleoprotein complexes in SV40 infected

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cells has shown an SV-40 specific 55 S complex. The protein portion of this complex is presently being analyzed by polyacrylamide gel electrophoresis. The complex isolated from cells infected with SV40 temperature sensitive mutants is also being examined (M. Zweig and N. Salzman).

In other studies, the single-stranded regions on superhelical SV40 DNA have been localized and characterized. We have studied the reaction product of SV40 DNA I with N-cyclo-hexyl-N-B-(4-methyl morpholinium)-ethylcarbodiimide (CMEC). This reagent reacts preferentially with single stranded regions of DNA. The product of the reaction, SV40 I-(CMEC), has an Sw,20 of 22.5S which is higher than SV40 DNA I (21S) and it also shows a small decrease in buoyant density. When ^{14}C-CMEC is reacted with ^{3}H SV40 DNA I, the reaction product can be cleaved by the restriction endonuclease R·Hin and the bound ^{14}C-CMEC is found associated with only three of the eleven cleavage fragments, namely fragments A, B, and G. The degree of single strandedness in these three fragments is estimated to be 7%, 4%, and 2.8% respectively. When either SV40 DNA I or SV40 DNA I-(CMEC) is cleaved by the single strand specific nuclease of N. crassa, the population of fragments that is obtained is consistent with cleavage at three sites at approximately 0.2, 0.45, and 0.55 genome lengths from the Eco R_I cleavage site. Fragment G contains the termination site of SV40 DNA replication and fragments A and B map with the in vivo initiation and termination of transcription of SV40 DNA. These single stranded regions in supercoiled SV40 DNA perhaps play an important role in SV40 DNA replication and transcription (M. Chen and J. Lebowitz).

In still other studies, the DNA of another papovavirus, polyoma, has been cleaved with the restriction enzyme, R·Hin. The DNA from small plaque polyoma strains yield 4 specific fragments representing 61%, 26%, 8%, and 5% of the original DNA. The 4 fragments produced by R·Hin have been ordered by analysis of partial digest products which were eluted and redigested with excess enzyme. Although the small plaque and large plaque strains of DNA both give 4 specific fragments after R·Hin digestion, the two strains have different biological activity. Moreover, when DNA's from small and large plaque strains of polyoma were cleaved with the Eco·R_I restriction enzyme, which gives only one cut to both strains, they form a specific heteroduplex region after denaturation and reannealing (M. Chen and N. Salzman).

In order to study the gene functions of the SV40 genome, it is important to localize the site of the mutation in different SV40 temperature-sensitive mutants. Several methods have been used to achieve this purpose. In our laboratory we have investigated these ts sites by looking for an anomalous electrophoretic migration of hybrid DNA molecules composed of mutant and wild type strands of R·Hin fragments. We have found such hybrids to possess altered electrophoretic migrations (M. Chen and N. Salzman).

Significance to Biomedical Research and the Program of the Institute:

Through the study of the structure-function relationships in small, genetically simple oncogenic viruses we hope to further define what properties of these viruses cause cells to integrate foreign DNA into their genome and to undergo

a malignant transformation. An understanding of these properties would shed considerable insight on the causes, prevention, and cure of naturally occurring malignancies.

In addition, since cellular functions play a major role in the replication of these small DNA viruses, it is hoped that the study of viral DNA replication will elucidate mammalian cellular mechanisms involved in cellular DNA replication and repair.

Proposed Course of the Project:

The structure-function relationships of SV40 DNA will be elucidated. The steps in DNA replication will be further defined.

Honors and Awards:

Editor, Journal of Virology
Visiting Scientist, University of Geneva, Geneva, Switzerland

Invited lectures at:

1. Cold Spring Harbor Lab, Cold Spring Harbor, N. Y.
2. St. Jude Children's Research Hospital, Memphis, Tennessee

Publications:

Unwinding of parental strands during Simian Virus 40 DNA replication. 1973. Salzman, N. P., Sebring, E. D., and Radonovich, M. J. Virol. 12:669-676.

The Mechanism of SV40 DNA replication. Salzman, N. P., Fareed, G. C., Sebring, E. D., and Thoren, M. M. Cold Spring Harbor Symposium on Quantitative Biology, Volume XXXVIII, 1974, Chromosome Structure and Function.

Superhelix density of replicating SV40 DNA molecules. 1974. Sebring, E. D., Garon, C. F., and Salzman, N. P. J. Mol. Biol., in press.

Serial No. NIAID-85A
1. Biology of Viruses
2. Biochemical Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Isolation and Characterization of Restriction Enzyme which Produce Unique Pieces of Viral DNA.

Previous Serial No.: Same

Principal Investigator: Dr. Frank DeFilippes

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	12/12			12/12
Professional:	12/12			12/12
Other:				

Project Description:

Objectives:

A) Develop an improved method of isolation of the restriction enzyme Hpa I from Hemophilus parainfluenzae. The method should be general enough so that it could be utilized for the isolation of other restriction enzymes and methyltransferases which act with the restriction system.

B) Characterize the Hpa I enzyme, especially with regard to its interaction with DNA.

C) To provide restriction endonucleases of Hemophilus parainfluenzae for collaboration within the laboratory for the study of SV40 viruses which are temperature sensitive and/or deletion mutants. Also to directly cleave the DNA of the SV40 host cell (Vero) to obtain unique fragments which may be separated into different size classes, and see if certain fragments are repeated throughout the amount of repetitive DNA associated with a given class of fragment size. This information might ultimately be used to see if some segments of cell DNA are more likely to include viral DNA in a linear array.

Also to cleave mitochondrial DNA, as an example of a small, normal cell DNA and to see if the unique fragment patterns for this DNA are changed by viral transformation.

Methods Employed:

A) and B) The enzyme was purified by standard biochemical techniques.

C) SV40 DNA, Vero cell DNA and mitochondrial DNA were purified by specific published procedures. The DNA is cleaved by the restriction enzyme and the fragments are separated, according to size, by gel electrophoresis to give characteristic patterns for each type of DNA.

Major Findings:

A) Purification of the Enzyme:

An alternate method of purification of the restriction enzyme Hpa I was developed which produced the highest yield of enzyme yet obtained. Relatively crude extracts, free of all contaminating deoxyribonuclease activity, with high levels of Hpa I activity, can be prepared by this method in two days and further purification of the enzyme which led to the highest specific activity yet found could be completed in several more days. The method utilizes two DEAE-cellulose columns which remove in sequence, nucleic acids and a second restriction enzyme as well as a potent exonuclease which acts upon double stranded DNA. The general features of the method make it attractive for the rapid isolation of a number of enzyme activities which either cleave SV40 DNA at specific sites, methylate it at specific sites or remove bases in the manner of exonuclease III from E. coli.

B) Characteristics of the Enzyme:

Sephadex chromatography in 1 M NaCl buffer indicates a molecular weight near 25,000 daltons. In low salt buffer, below pH 8.3 the enzyme aggregates and appears to have a molecular size greater than 100,000 daltons. Aggregation in low salt buffer is inhibited above pH 8.3 where enzyme activity is associated with several different size classes. The isoelectric point of the enzyme is near pH 8 as judged by its ability to bind to different ion exchange columns. The enzyme has an absolute requirement for Mg ion. Other divalent ions diminish enzymatic activity. The enzyme is most active in buffer containing 80 mM NaCl at pH 7.85 in the presence of 14 mM mercaptoethanol, although it will cleave DNA in the absence of salt and reducing agent. The cleavage reaction is not inhibited by either sRNA, rRNA or sonicated single stranded DNA when the competing nucleic acids are present in concentrations close to the substrate DNA concentration. The enzyme will however bind to both double stranded and single stranded DNA cellulose columns under non-enzymatic conditions (no Mg^{++} present) or with Mg^{++} present. Mg serves to slightly enhance the binding to both single and double stranded DNA. At pH 7.6, in the absence of Mg about 400 µg of double stranded DNA (in a cellulose column) is

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needed to bind an amount of enzyme which will cleave 10 µg of DNA substrate in one minute. The enzyme will bind to double stranded DNA, at this pH, in 100 mM NaCl, but it will not bind to single stranded DNA above 50 mM NaCl. In 50 mM NaCl it binds to double stranded DNA cellulose over a pH range of 6.9 to 8.9 and it will bind in the presence of sonicated single stranded DNA at pH 7.6. The enzyme is heat stable with or without its DNA substrate at 37° for at least 5 hours but it is inactivated within minutes at 60°C.

C) Cleavage of DNA by the Restriction Enzyme:

Hpa I cleaves SV40 DNA into 3 fragments, which are separated by gel electrophoresis. The largest fragment has a size of about 1.5×10^6 daltons. The same enzyme cleaves very large DNA (about 150×10^6 daltons) prepared from the virus host cell (Vero) into unique fragments which after separation by gel electrophoresis show a continuous distribution of piece sizes, with an indication of small peaks superimposed on the distribution. The gel pattern was assymmetric with a peak fragment size being larger than the largest size fragment of SV40 DNA.

Another class of normal cell DNA, mitochondrial DNA (M-DNA) was digested with the restriction enzyme and analyzed by gel electrophoresis. M-DNA from different organs of the same animal shows the same pattern. Patterns for different animals were different. In all cases 5 or more fragments were obtained. After alkali denaturation the single stranded DNA was reduced to small pieces corroborating recent work indicating the presence of ribonucleotides in the mature DNA.

Significance to Bio-medical Research and the Program of the Institute:

The isolation and characterization of restriction enzymes has given great impetus to understanding the structure and function of viral DNA. The enzymes provide a means for isolating specific genes from viral and mitochondrial DNAs and of providing a unique set of fragments from the genome of eukaryotic cells.

Proposed Course of the Project:

Specific DNA methylases associated with the restriction enzymes from Hemophilus parainfluenzae will be isolated and tested to see if they provide a unique means for labeling SV40 DNA.

DNA from Vero cells and SV40 and polyoma transformed cell lines will be cleaved by the restriction enzyme and the different size classes tested for the amount of unique and repeated DNA present.

Mitochondrial DNA will be examined to see if some cases of transformation produce alterations in the cleavage pattern of this DNA.

Publications:

DeFilippes, F.: A new method for isolation of a restriction enzyme from hemophilus parainfluenzae. Biochem. Biophys. Res. Commun.: in press.

Serial No. NIAID-87A
1. Biology of Viruses
2. Molecular Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Properties of the Nucleic Acid and Protein Components of Adenovirus-Associated Viruses

Previous Serial Number:

Principal Investigator: Dr. James A. Rose

Other Investigators: Dr. Barrie J. Carter
Dr. Claude Garon
Dr. Steven Straus
Mr. Frank Koczot
Mr. James Garrison

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	39/12		6/12	45/12
Professional:	21/12		6/12	27/12
Other:	18/12			18/12

Project Description:

Adenovirus-associated viruses (AAV) are small, DNA-containing viruses multiply only in cells which are co-infected with an adenovirus. Herpesviruses also provide some function(s) required for AAV multiplication; AAV antigen(s) is synthesized but an increase in AAV infectivity or particles has not been observed. Four AAV serotypes are now known. We have shown previously that AAV DNA is segregated in a way not as yet observed with any other virus. Single, complementary DNA strands are separately encapsidated in different virions. On extraction of viral DNA, the complementary strands anneal to form duplex molecules. There are two species of complementary DNA strands which can be separated in CsCl density gradients. Studies are directed toward the biological and biochemical characterization of AAV infection.

Objectives:

1. To analyze the structure and function of the complementary strand species of AAV-DNA.

2. To identify the helper virus-mediated step(s) in the AAV replication sequence.

3. To analyze the structure and function of *in vivo* synthesized AAV RNA species induced by either partial or complete helper viruses.

Methods Employed:

The AAV are produced in KB cells in spinner culture. Viruses are purified in CsCl density gradients, and DNA is extracted from purified virus by treatment with alkali or with proteolytic enzymes, sodium dodecyl sulfate and phenol. The complementary strand species are separated by centrifuging denatured, bromodeoxyuridine-substituted DNA to equilibrium in neutral CsCl density gradients. Viral RNA synthesized *in vivo* or *in vitro* is labeled with ^{3}H or ^{32}P. DNA/DNA or DNA/RNA complementarity is determined by nucleic acid hybridization and hybrids are detected on nitrocellulose filters, in CsCl density gradients or by hydroxyapatite chromatography. RNA is further analyzed by sedimentation in dimethylsulfoxide sucrose gradients and electrophoresis in acrylamide gels. DNA molecules are directly visualized in the electron microscope using either aqueous or formamide spreading techniques.

Major Findings:

1. Viral RNA analyses revealed two populations of AAV RNA, (i) a single, discrete 20S species and (ii) a very heterogeneous population ranging in size from 4S to 18S. In the nucleus both populations of AAV RNA were observed but in the cytoplasm the 20S RNA was mainly present in the polysome fraction and the heterogeneous RNA was mainly in the non-polysomal fraction. The 20S RNA, therefore, appears to be the only functional AAV message species. The heterogeneous AAV RNA may arise from incomplete transcription or degradation of the 20S RNA in the nucleus or cytoplasm. The molecular weight of the 20S AAV RNA was approximately 0.9 to 1.0 x 10^6 which accounts for the entire region of the AAV genome that is stably transcribed. This 20S RNA may be monocistronic and thus AAV DNA may contain only one gene.

The 20S AAV RNA did not appear to be formed by post-transcriptional cleavage of a larger nuclear precursor. In the same experiments the post-transcriptional cleavage of Ad RNA was clearly observed. At least nine species of Ad RNA were present in the cytoplasm late after infection and at least six of these were associated with polysomes. Non-polysomal, cytoplasmic Ad RNA was heterogeneous but contained one discrete, 5.5S species (VA RNA) which was transported to the cytoplasm more rapidly than either Ad or AAV message RNA or cellular rRNA.

2. When heteroduplexes were prepared with any combination of AAV-1, -2 or -3 DNA, regions containing heterology melted out progressively as denaturing conditions were increased. This indicates that partial homology is present within these regions, and suggests that sequence differences among the serotypes have arisen by retention of random base changes. In addition, interserotypic homologies physically mapped at similar locations, indicating that the same sequences are conserved in all three serotypes. No melting was observed in homoduplex molecules spread under similar conditions. Because single-stranded circles remain essentially intact under the same spreading conditions, open heteroduplex ends mean that the terminal self-complementary sequences are not completely conserved among different serotypes.

3. When heteroduplex molecules, prepared with any combination of AAV-1, -2, or -3 DNA, were spread in high concentrations of formamide, self-annealed strand ends could be specifically located and "panhandle" structures readily identified. These projections confirmed the mode of terminal base arrangement which previously had been uncertain. From the length of these projections it was estimated that the terminal repetition in AAV DNA is equivalent to 4-8% of total strand DNA (100-200 base pairs).

4. Based on DNA/DNA competition hybridization at least 60-70% of sequences in AAV-1, -2 and -3 DNA have been found to be homologous, but heteroduplex melting studies suggest that interserotypic homologies actually approach 90-95%.

Significance to Biomedical Research and the Program of the Institute:

Human infection with AAV has been shown to be frequent, but its significance is not yet known, since a virus-specific clinical disease has not been defined. Characterization of biochemical and biological properties of these viruses may be important with respect to human disease, because 1) although now inapparent, they might be human pathogens and, 2) the mechanism(s) by which helper viruses assist AAV replication are relevant to understanding natural modes of viral infection and may be important with respect to the development of biochemical controls of virus replication. In contrast to cell DNA, the relatively small genomes of these viruses permit more comprehensive biochemical and physical analyses of function and structure. For example, our discovery of a new type of genetic structure at the ends of both the adenovirus and AAV genomes (inverted terminal repetition) may help to explain the mechanism of DNA synthesis for both viruses as well as represent (at least partially) the molecular reason for AAV defectiveness. Because this terminal structure appears to be highly specific, further definition of its biochemical properties could provide an important basis for identifying or preparing compounds with selective antiviral activity. In addition, studies on the biochemical dependence by AAV for specific adenovirus gene products may lead to a means for purifying and characterizing these control substances. This, in turn, could provide insight into basic properties of gene regulation in general as well as offer new approaches in the development of antiviral agents.

Proposed Course of the Project:

Continue to study AAV replication and defectiveness.

Honors and Awards:

Invited participant at:

1. Gordon Research Conference on Animal Cells and Viruses
2. Cold Spring Harbor meeting on Adenoviruses
3. 1974 Cold Spring Harbor Symposium
4. Elected to American Society for Clinical Investigation

Publications:

Carter, B. J. and Rose, J. A.: Transcription in vivo of a defective parvovirus: Sedimentation and electrophoretic analysis of RNA synthesized by adenovirus-associated virus and its helper adenovirus. Virology, in press.

Rose, J. A.: Parvovirus reproduction, in "Comprehensive Virology", (H. Fraenkel-Conrat and R. Wagner, ed.) Plenum Press, New York, in press.

Serial No. NIAID-87B
1. Biology of Viruses
2. Molecular Structure
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Properties of Adenovirus DNA

Previous Serial No.: Same

Principal Investigators: Dr. Claude F. Garon
Dr. James A. Rose

Other Investigators: Ms. K. W. Berry
Mr. James Garrison
Dr. G. Lavelle

Cooperating Units: Dr. Robert F. Friedman, Laboratory of Pathology, NCI
Dr. Robert Stern, Laboratory of Biochemistry, NIDR

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	39/12			39/12
Professional:	21/12			21/12
Other:	18/12			18/12

Project Description:

Objectives:

To provide a detailed map of base sequence homologies and heterologies among the genomes of several oncogenic and nononcogenic adenovirus groups. And, further, to correlate mappable sequences with a biochemical characterization of both structural and replicative properties of these viruses.

Methods Employed:

Viruses are produced in KB cells and purified in CsCl equilibrium density gradients. Viral DNA is extracted from purified virus by treatment with proteolytic enzymes, sodium dodexylsulfate and phenol. The electron microscope heteroduplex method consists of constructing in vitro a molecule in which one strand of the DNA duplex is derived from each of the viruses being compared. Using the formamide modification of the basic protein film procedures for visualiza-

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tion of the DNA, regions of sufficient homology form duplex structures, while regions containing heterologous sequences remain as recognizably unpaired single strands. Furthermore, varying by formamide concentration (isodenaturation procedure) regions of the genome containing partial homology may be identified. careful measurement of these structural features reveal both the extent and map location of sequence heterology between the viruses being compared. Heteroduplexes may be effectively purified using benzoylated-naphthoylated DEAE-cellulose (BND) column chromatography and specifically fragmented using single-strand specific nucleases. Viral DNA forms are also analyzed by hydroxyapatite chromatography, sucrose gradient sedimentation and agarose gel electrophoresis.

Major Findings:

1. Electron microscopic studies with DNA from a number of human adenovirus serotypes have revealed that single-stranded, circular molecules are formed when native DNA is denatured and then annealed. Owing to the presence of an inverted terminal repetition the ends of denatured DNA molecules can self-anneal to form the observed single-stranded circles closed by a duplex hydrogen-bonded segment. Although the arrangement of sequences within the repetition should be apparent from the morphology of the closure region, this region had been too short to visualize by electron microscopy. However, in contrast to the circular molecules produced with DNA from all other serotypes, there was associated with each Ad 18 circular molecule a visible, apparently duplex, projection of 0.31 ± 0.12 μ in length representing approximately 6% of the genome. Occasionally longer duplex projections have also been observed. Alkaline sucrose gradient purification of native molecules did not alter formation of these projections. Treatment of a preparation of circular molecules with N. crassa, single-strand specific nucleases resulted in the production of 0.34 ± 0.09 μm duplex fragments. Single-stranded circles do not form if a limited number of nucleotides are removed from the 3' ends of the native molecule by E. coli exonuclease III digestion prior to denaturation and annealing. Preformed single-stranded circles could be converted to linear molecules by similar treatment. The duplex closure region of circular molecules showed considerable heat stability requiring a temperature equivalent above the Tm of native DNA to initiate melting. We therefore conclude that Ad 18 DNA has an unusually large inverted terminal repetition and that single-stranded circles are closed by a duplex antiparallel base-paired "panhandle." Length measurements of circular molecules and the detection of specific heteroduplexes predicted from an annealing of strands which contain terminal repetitions of different lengths support the view that 1) repetitious sequences occur in place of rather than in addition to regular sequences and 2) at least those molecules with relatively long repetitions should represent defective genomes.

2. Heteroduplex DNA molecules, prepared by hybridizing DNA of adenovirus serotypes 1, 2 and 5 in all three combinations, have been shown by electron microscopy to contain two specifically located mismatched regions. Single-strand specific nucleases from N. crassa cleaved the heteroduplexes into three fragments as demonstrated by sucrose gradient analysis, agarose gel electrophoresis and electron microscopy. These were produced in equimolar amounts, and are shown by alkaline sucrose gradient analysis to be free of

internal single-strand interruptions. The size of each of the fragments was determined by sucrose gradient analysis and by electron microscopic measurements of the contour lengths. The three values obtained correspond to the lengths of the three double-stranded regions in the untreated heteroduplex molecules. The fragments, free of detectable single-stranded tails have been isolated preparatively from neutral sucrose gradients. Renaturation kinetic analyses support the conclusion that each fragment represents a unique segment of the adenovirus genome.

3. Single-stranded fragments of adenovirus type 2 DNA were isolated from infected KB cells under conditions which retarded reassociation of complementary sequences but did not denature native viral DNA. Of the total intracellular, virus-specific DNA, recovered from cells at high efficiencies after a 1 hr pulse with ^{3}H-dThd beginning 15 hr after infection, about 20% was single-stranded when fractionated on hydroxyapatite. This DNA shifted to the double-stranded fraction on hydroxyapatite during an extended chase incubation suggesting the presence of single-stranded DNA in replicating intermediates. Furthermore, single-stranded DNA annealed nearly equally to both strands of the adenovirus genome. These findings are consistent with a model of DNA replication involving strand-displacement with synthesis of the displaced strand being initiated from either end of the parent template.

4. Phleomycin, a polypeptide with antibiotic and anti-tumor activity, produces single-strand breaks in purified adenovirus and phage λ deoxyribonucleic acid. A reducing agent was required and both native and single-stranded deoxyribonucleic acids were substrates for this activity. The limit reaction products were acid insoluble and sedimented at 5 S or less in sucrose density gradients. The deoxyribonucleic acid cleaving activity of phleomycin could be measured by a nitrocellulose filter binding assay and characteristics of this assay are described. Ribonucleic acid did not appear to be cleaved by phleomycin.

5. Phleomycin stimulates the incorporation of thymidine triphosphate (TTP) into DNA in isolated nuclei from several animal cell lines; however, phleomycin also induces degradation of nuclear DNA as judged by alkaline sucrose density gradient centrifugation. The stimulation of TTP incorporation by phleomycin requires Mg^{2+} and deoxynucleoside triphosphates. In addition, phleomycin activity has considerable heat stability.

It is suggested that stimulation of TTP incorporation by phleomycin may be related to an increase in DNA synthetic activity secondary to extensive strand breakage induced by the drug.

Significance to Bio-Medical Research and the Program of the Institute:

The added dimension provided by the electron microscope heteroduplex mapping method over DNA-DNA hybridization techniques is the ability to localize (possibly within several nucleotide bases using techniques developed in this laboratory) regions of homology and non-homology along the contour length of an intact molecule. Furthermore, the treatment of purified heteroduplex molecules with single-strand specific nucleases provides a procedure for generating preparative quantities of both physically and genetically defined fragments by

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a method that does not depend on the recognition of sequence-specific cleavage sites. The procedure is potentially applicable to the fractionation of many double-stranded, polynucleotide molecules. Such genetically defined fragments could be of help in locating sites having specific functions in viral genomes which in turn may be necessary to understanding how viruses influence or gain control of cellular functions.

Proposed Course of the Project:

1. To continue using the electron microscope heteroduplex mapping technique to provide direct structural characterizations of genomes from members of several oncogenic and nononcogenic adenovirus groups.

2. To correlate genetic maps with biochemical functions expressed during viral infection.

3. To define in molecular terms the basis for genetic interactions between viruses and cells.

Publications:

Garon, C. F., Berry, K. W., Hierholzer, J. C., and Rose, J. A.: Mapping of base sequence heterologies between genomes from different adenovirus serotypes. Virology 54: 414-426, 1973.

Stern, R., Rose, J. A. and Rose, J. A.: Phleomycin-induced cleavage of deoxyribonucleic acid. Biochemistry 13: 307-312, 1974.

Friedman, R. M., Stern, R., and Rose, J. A.: Phleomycin stimulation of thymidine triphosphate incorporation by animal cell nuclei. J. Nat. Cancer Inst. 52: 693-697, 1974.

Bartok, K., Garon, C. F., Berry, K. W., Fraser, M. J., and Rose, J. A.: Specific fragmentation of adenovirus heteroduplex molecules with single-strand specific nucleases of Neurospora crassa. J. Mol. Biol., in press.

Serial No. NIAID-88
1. Biology of Viruses
2. Physical Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on the Biosynthesis and Characterization of Proteins in SV40 Transformed and Infected Cells

Previous Serial No.: Same

Principal Investigator: Dr. Stanley Barban

Other Investigators:

Cooperating Units: Dr. Ettore Appella, LCB, NCI

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned To Project	Guest Workers	Total
Total:	12/12			12/12
Professional:	12/12			12/12
Other:				

Project Description:

Objectives:

1. Isolation, identification, and characterization of the proteins specified by the SV40 viral genome early in the infectious cycle.

2. To examine and characterize the proteins that are associated with the viral DNA in infected and transformed cells.

3. To analyze the peptide and amino acid compositions of the structural proteins of SV40 mutants.

Methods Employed:

Simian virus (SV40) was grown in primary and continuous green monkey cells and purified by density gradient sedimentation. Biosynthesis of protein macromolecules was determined by incorporation of radioactive precursors. The labeled molecules were isolated and purified by gel-electrophoresis,

column chromatography, isoelectric focusing, differential zonal centrifugation, immunoabsorption and immuno-precipitation. Structural analyses of protein subunits was performed by enzymatic and chemical hydrolysis followed by chromatographic and electrophoretic separation. Cell fractionation techniques was used to isolate subcellular components.

Major Findings:

A. Isolation and identification of virus-induced proteins in SV40 infected cells. Immunosorbent affinity chromatographic techniques was used to isolate from infected cells virus specific proteins. Identification of the labeled proteins was made by analytical polyacrylamide gel electrophoresis and immunochemical analyses. Pulse-label experiments showed that approximately 5 to 10% of the polypeptides synthesized after infection are identical to the two major structural proteins of the virion, the coat proteins. These appear after the onset of viral deoxyribonucleic acid synthesis. Prior to the viral DNA synthesis, and in cells in which viral DNA is inhibited by cytosine arabinoside there appears two polypeptides which are virus-induced and are not virion structural proteins. One of the two proteins has an estimated molecular weight of 70,000 daltons. These non-structural proteins have not been fully characterized.

Subunit Composition of the SV40 capsid proteins. Previous studies suggested that the main coat protein of SV40 is composed of two identical subunits. Additional evidence has now been obtained by amino acid analyses and peptide mapping technique to substantiate the earlier findings.

Significance to Biomedical Research and the Program of the Institute:

The ultimate goal of these studies is the isolation and characterization of those proteins specified by the SV40 viral genome which could be related to viral transformation or integration into the host cell genome. Studies on the proteins involved in virus-induced transformation, biosynthesis of the early antigens especially the tumor and transplantation antigens are of special interest in the study of tumor biology. An understanding of the proteins or gene products involved in viral-transformation may enable us to regulate or control their biosynthesis by immunological or appropriate chemotherapeutic agents.

Proposed Course of the Project:

Emphasis will be placed on developing specific immunoabsorbents and immunological probes for the isolation and characterization of those non-structural proteins specified by the viral genome. Infected cells in which viral replication is blocked by metabolic inhibitors will be used to delineate and simulate conditions of the transforming events to study the SV40 specified proteins. Further studies of the proteins found in association with the viral DNA in lytic and transforming infection will be continued.

Honors and Awards:

None

Publications:

Barban, S.: Electrophoretic differences in the capsid proteins of Simian Virus 40 plaque mutants. J. Virol., 11:971-977, 1973.

Lake, R. S., Barban, S., and Salzman, N. P.: Resolutions and identification of the core deoxynucleoproteins of the Simian Virus 40. Biochem. Biophys. Res. Comm., 54:640-647, 1973.

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Serial No. NIAID-88B
1. Biology of Viruses
2. Physical Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Biochemistry of Chromatin and Nucleic Acids

Previous Serial Number: Same

Principal Investigator: Dr. Thomas Y. Shih

Other Investigators: Dr. Malcolm Martin
Dr. George Khoury

Cooperating Unit: Dr. H. J. Li, Department of Chemistry, Brooklyn College of the City, University of New York

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	18/12			18/12
Professional:	18/12			18/12
Other:				

Project Description:

Objectives:

To study the chemistry and biology of nucleic acids and chromatin, and their relationship to the regulation of viral gene expression during transforming and lytic infection.

Methods Employed:

A. Organic synthetic methods were employed to covalently link viral DNA or RNA to cellulose derivatives for affinity chromatography.

B. Molecular hybridization was employed to study the template function of the integrated SV40 genome in chromatin of virus transformed cells.

C. Thermal denaturation and circular dichroism were employed to study the structure and interactions of DNA-protein complexes.

Major Findings:

A. Covalent linkage of nucleic acid to cellulose. DNA was chemically immobilized to neutral cellulose powder using 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide p-toluenesulfonate (CMC). Under the conditions developed, 45-78% of fragmented SV40 DNA could be stably linked to the cellulose matrix presumably through phosphodiester bonds involving the terminal phosphate group of DNA and hydroxyl groups of the neutral cellulose SV40 complementary RNA (cRNA), synthesized in vitro with E. coli RNA polymerase was also securely immobilized onto phosphocellulose powder activated by 1,1'-carbonyldiimidazole.

We have been able to isolate complementary DNA or RNA sequences from reaction mixtures containing a vast excess of unrelated sequences by employing DNA or RNA cellulose columns in a continuous hybridization system. From thermal stability studies it was concluded that the selective isolation of viral nucleic acid involved the formation of specific hybrid structures between the circulating labeled and immobilized, unlabeled, nucleic acid species.

B. The template function of chromatin of SV40 transformed cell was studied in an in vitro transcription system involving E. coli RNA polymerase chromatin is a restrictive template, the integrated viral sequences were transcribed and shown to be complementary to the minus strand of SV40 DNA.

C. Conformation of DNA in chromatin is altered due to association with histones. Since the slightly lysine rich histone is the major protein component of chromatin, the DNA complexes of the pure histone IIb2 was studied by the method of thermal denaturation and circular dichroism. It was found that the pure histone induces conformational changes of DNA similar to those found in chromatin.

Significance to Biomedical Research and the Program of the Institute:

Development of the affinity chromatographic methods for specific viral DNA or RNA sequence isolation could potentially add to our understanding of the molecular processes involved in viral infection and transformation. Studies on the transcription of integrated viral sequences in chromatin of transformed cells could provide better understanding on the molecular basis of gene transcription, especially the molecular control of viral gene activity.

Proposed Course of the Project:

To explore the applications of the affinity chromatographic methods in virus research, and to study the transcription of the integrated viral sequences of chromatin.

Honors and Awards:

Invited lecturer at the Brooklyn College of the City University of New York in December 1973.

Publications:

Shih, T. Y. and Martin, M. A.: A general method of gene isolation. Proc. Nat. Acad. Sci., 70:1697-1700, 1973.

Shih, T. Y., Khoury, G., and Martin, M. A.: In vitro transcription of the viral-specific sequences present in the chromatin of cells transformed by simian virus 40. Proc. Nat. Acad. Sci., 70:3506-3510, 1973.

Leffak, I. M., Hwan, J. C., Li, H. J., and Shih, T. Y.: Circular dichroism and thermal denaturation studies of nucleohistone IIb2. Biochemistry, 13: 1116-1121, 1974.

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LABORATORY OF MICROBIAL IMMUNITY

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PHS-NIH
Summary Statement
Laboratory of Microbial Immunity
July 1, 1973 through June 30, 1974

Research in this Laboratory concerns the control and expression of immunity to microbial and tissue antigens. Research has progressed in several areas: (1) Functionally active thymus-derived (T) cells can be separated from each other. (2) T-cells which produce specific suppression of an immune response to a bacterial polysaccharide can be activated by specific antigen. (3) A new molecule, dinitrophenyl-ficoll, is being used to investigate activation and paralysis of potential antibody forming cells. (4) Graft-versus-host reactions differ in reciprocal hybrid mice of the same genotype suggesting that factors other than genotype can determine the outcome of such reactions, a finding that may ultimately have therapeutic importance. (5) Athymic "nude" mice appear to be very susceptible to infection by certain viruses and develop a disorder similar to human lupus erythematosis.

Major changes occurred in LMI in the past year. The germfree animal unit, together with four staff positions, were abolished. A planned expansion of laboratory and conventional animal space was made impossible by the installation of an insectary in the area formerly occupied by the germfree unit. The Laboratory is supposed to move to Building 5 in the coming year, where the needed space exists.

Dr. Robert E. Tigelaar returned from a productive working assignment in London. He learned methods for in vitro sensitization and fractionation of lymphocytes. This year he became a member of the permanent staff of LMI. Dr. John Finerty is on working assignment at the Trudeau Institute, Saranac Lake, New York. He is studying induction cellular immunity to blood parasites. Dr. Jeffrey M. Jones joined the Laboratory this year as a research associate. He has been working with Dr. Phillip J. Baker on immune responses to Type III pneumococcal polysaccharide.

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RESEARCH ACCOMPLISHMENTS

TWO FUNCTIONS OF THYMUS-DERIVED (T) CELLS SEPARATED

The immune response of C57BL mice to a DBA/2 tumor allograft has been assessed in two assays of cell-mediated immunity, the *in vitro* lysis of ^{51}Cr-labelled target cells and the antigen-mediated inhibition of macrophage migration. Both assays were shown to be measuring a T cell-mediated reaction. Three types of experiments suggested that distinct subpopulations of T cells mediate these reactions. The subpopulations have (1) distinctive tissue distributions in appropriately immunized animals, (2) differential migratory properties upon adoptive transfer into irradiated syngeneic hosts, and (3) distinctive activity profiles after 1g velocity sedimentation. In addition, normal spleen cells were fractionated by velocity sedimentation immediately prior to allosensitization *in vitro*. Subsequent analysis of the sensitized fractions revealed that the activity profiles for cytotoxicity and macrophage migration inhibitation were not coincident, suggesting that the progenitors of these T effector cells may be different. These results give further support to the concept that the pool of thymus-derived lymphocytes is composed of subpopulations of cells, each with discrete and limited functional capabilities. (Drs. Tigelaar and Gorzcynski (University College, London))

SUPPRESSOR "T" CELLS MEDIATE LOW-DOSE PARALYSIS

Prior treatment with very low doses of Type III pneumococcal polysaccharide (SSS-III) greatly reduces the capacity of mice to respond to subsequent immunization with this antigen. Such low-dose paralysis, which is antigen-specific, could not be induced in the absence of thymic-derived cells (T cells) and could be abrogated by treatment with antilymphocyte serum (ALS), a procedure reported to inactivate suppressor T cells. These findings suggest that low-dose paralysis to SSS-III is a T cell dependent phenomenon in which suppressor T cells, activated by low doses of antigen, play a major role. (Dr. Baker, Mr. Stashak, and Miss Amsbaugh, with Dr. Prescott, Laboratory of Microbiology, NIAID)

PROPERTIES OF A HAPTEN-CONJUGATED POLYSACCHARIDE STUDIED

Studies upon the induction of tolerance have indicated that the hapten dinitrophenol can induce tolerance *in vitro* when coupled to a sugar carrier, Ficoll. The number of haptens per unit volume of the culture is more important than the number of carrier molecules in the process. The rapidity of induction of tolerance was faster when the number of haptens per carrier molecule was a large number. The process occurred too rapidly to be accounted for by the normal decay of messenger RNA, yet left the cells alive as judged by a dye exclusion test. DNP-ficoll can also be used to induce immunity *in vitro* if very low doses are employed. (Drs. McMaster, Bachvaroff (not NIH), and Rappaport (not NIH))

GRAFT-VERSUS-HOST (GVH) REACTIONS IN RECIPROCAL HYBRID MICE STUDIED

Spleen cells from BALB/c and C57BL/6 mice were tested for their reactivity against reciprocal hybrid tissues ((BALB/c x C57BL/6) F_1 and (C57BL/6 x BALB/c) F_1) in three assay systems--the

mixed lymphocyte reaction (MLR), the Simonsen spleen-weight GVH assay, and a GVH mortality assay. It was shown that both F_1's serve as equally effective stimulators of parental cells in the MLR. In the spleen-weight assay, BALB/c and C57BL/6 cells were equally active in a given host, but greater splenomegaly was observed in (BALB/c x C57BL/6) F_1 hosts regardless of the donor strain. By contrast, BALB/c cells were much less lethal than C57BL/6 cells in (BALB/c x C57BL/6) F_1 hosts than in (C57BL/6 x BALB/c) F_1 hosts, and to a lesser degree C57BL/6 cells were less lethal than BALB/c cells in (C57BL/6 x BALB/c) F_1 hosts. The possibilities exist that modifying substances may differentially alter reactivity of parental lymphocytes and that considerations other than genotype determine the outcome of a GVH reaction. (Drs. Morse, Harrison (LI), and Asofsky)

"AUTOIMMUNE" DISEASE IN ATHYMIC MICE Sera from one- to four-month-old congenitally athymic "nude" mice and their phenotypically normal littermates (LM) were tested for their capacity to bind double stranded DNA and RNA. Sera from nudes bound significantly more DNA than did LM sera with no differences in RNA-binding capacity being demonstrable. Decline in the clinical state of nude mice was associated with increased serum DNA-binding capacity. Nude mice grafted with thymi at one month of age had reduced serum DNA-binding capacities when compared to non-grafted nudes. Immunofluorescent staining of kidneys from nude mice revealed the presence of significant amounts of IgM, IgG_1, IgG_2, and IgA immunoglobulins in the glomeruli of nudes but not LM mice. In some cases, immunoglobulin was associated with complement (β_{1c}) deposition. These findings may be explained by the absence of suppressor cells or by the existence of chronic infection in athymic mice. These mice often have chronic hepatitis, and mouse hepatitis virus can be isolated from their livers. (Drs. Morse, Steinberg (A), Schur (not NIH), and Cross (not NIH))

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Serial No. NIAID - 110
1. Microbial Immunity
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Administration, research planning and coordination

Previous Serial Number: Same

Principal Investigator: Richard Asofsky

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	14/12			14/12
Professional:	2/12			2/12
Other:	12/12			12/12

Project Description:

Administrative and supervisory support is provided to all personnel and to each project of the Laboratory.

Research plans are discussed with the various investigators, over-all evaluations made and the research activities of the Laboratory coordinated. Collaborative research with specialists in other laboratories of NIAID, or institutes of the NIH, or various university investigators is encouraged and assistance given the professional staff in effecting the necessary arrangements. Technical consultation is provided to individuals of the NIH, and to scientists of other departments of the Government or academic organizations. Assistance is provided the professional staff in the preparation of manuscripts and in editing and reviewing a variety of technical reports. The needs of all personnel are evaluated and an effort made to obtain the necessary equipment and supplies for the efficient conduct of research. Budget requirements are forecast on a yearly basis and emergency needs dealt with as problems arise. Supervision is given in regard to laboratory expenditures, travel of personnel, and correspondence. When possible, highly qualified professionals and technicians are sought as staff additions or replacements to insure a high level of research excellence.

Serial No. NIAID - 111-B
1. Microbial Immunity
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on the pathogenesis of amebic enteritis

Previous Serial Number: None

Principal Investigator: Bruce P. Phillips

Other Investigators: Akio Takeuchi

Cooperating Units: Department of Experimental Pathology, Walter Reed Army Institute of Research, Washington, D.C.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	4/12			4/12
Professional:	4/12			4/12
Other:	--			--

Project Description:

Objectives:

To observe and record the microscopic alterations and events associated with the penetration of enteric tissues by Entamoeba histolytica including response of tissue including its microcirculation to amebic invasion.

Methods Employed:

Germfree guinea pigs are inoculated with E. histolytica and the accompanying enteric flora from acute cases of amebic enteritis in humans. At various intervals after inoculation, the animal is anesthetized with ethyl carbamate and a newly-developed method for microscopic observation of the cecal mucosa and its microcirculation in the living anesthetized animal is employed for observing changes associated with pathogenesis of the disease. An ever present 16 mm movie camera is used to record tissue events which occur by time-lapse photomicrography.

Major Findings:

The first phase of the studies has been reported. This concerned the mechanism(s) of tissue penetration by ameba. The current phase is new and

much effort has gone into development of the complex technical procedures involved. Observations thus far have been limited to those involving early effects on the microcirculation with stagnation and thrombosis together with some increase in wandering histiocytes but no direct observations as yet of amebic penetration of epithelium.

Significance to Biomedical Research and the Program of the Institute:

Although we have previously observed and reported the first detailed study of mechanisms of tissue invasion in amebic enteritis based on large scale (EM) fine structure observations, it is important in this ubiquitous disease to learn as much as possible about the microscopic events associated with its pathogenesis.

Proposed Course of Project:

This research will be discontinued as the result of reduction in program.

Honors and Awards: None

Publications:

Takeuchi, A., and Phillips, B. P.: Electron microscopic studies of Entamoeba histolytica infection in the guinea pig. I. Penetration of the intestinal epithelium by trophozoites. Am. J. Trop. Med. and Hyg. In press.

Serial No. NIAID - 111-C
1. Microbial Immunity
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Investigations on Blastocystis hominis in germfree and controlled-flora animals

Previous Serial Number: Same

Principal Investigator: Bruce P. Phillips

Other Investigators: Charles H. Zierdt

Cooperating Units: Laboratory of Clinical Pathology, Clinical Center, NIH, Bethesda, Maryland

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	2/12			2/12
Professional:	2/12			2/12
Other:	--			--

Project Description:

Objectives:

To ascertain whether Blastocystis hominis from symptomatic cases of enteritis possibly attributable to this organism does indeed have pathogenic potential and, if so, under what conditions.

Methods Employed:

Blastocystis hominis and its accompanying enteric flora from Clinical Center patients with enteric symptoms of undiagnosed etiology is introduced intracecally into germfree guinea pigs. Animals are observed for symptoms and are sacrificed at various intervals and examined for enteric tissue changes and for degree of B. hominis infection in the gut.

Major Findings:

We previously showed the B. hominis could be freed of its accompanying flora with a combination of eight antibiotics, but it would not survive in the gut of the germfree guinea pig. More recently, success has been achieved in cultivating this protozoan axenically in pre-reduced medium (it is an obligate

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anaerobe) and these organisms have likewise shown an inability to survive in the germfree gut (which has a high redox potential). The organism has also exhibited an inability to establish infections and grow in the intestine of conventional animals. However, recent experiments wherein B. hominis and its accompanying enteric flora from human patients were inoculated intracecally into germfree guinea pigs have produced many heavy infections. In several of these infections the animals have shown diarrheal symptoms and some weight loss. In one animal with a particularly heavy infection, the cecum showed a superficial erythema.

Significance to Biomedical Research and the Program of the Institute:

There have been a number of hospital patients in whom severe enteric symptoms could not be attributed to the presence of any known pathogens but who harbored heavy infections with B. hominis. It is important to ascertain if this protozoan, long erroneously classified as a yeast and considered harmless, does possess pathogenic potential and if so, under what conditions does it manifest pathogenicity.

Proposed Course of Project:

This research will be discontinued as the result of reductions in program.

Honors and Awards: None

Publications:

Phillips, B. P., and Zierdt, C. H.: Blastocystis hominis: An ameba with pathogenic potential. Proc. Assoc. Gnotobiotics, Guelph, Ontario, 1974. In press.

Serial No. NIAID - 111-G
1. Microbial Immunity
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The pathogenic determinants in Entamoeba histolytica with special reference to intracellular viruses of non-pathogenic and highly pathogenic strains

Previous Serial Number: None

Principal Investigator: Bruce P. Phillips

Other Investigators: Louis S. Diamond*
Carl F. T. Mattern**

Cooperating Units: *Laboratory of Parasitic Diseases, NIAID
**Laboratory of Virus Diseases, NIAID

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	2/12			2/12
Professional:	2/12			2/12
Other:	--			--

Project Description:

Objectives:

To ascertain why strains of morphologically identical Entamoeba histolytica vary to the extreme in their pathogenic potential and to identify the factor(s) responsible for this phenomenon.

Methods Employed:

Numerous strains of axenically cultivated E. histolytica are inoculated intrahepatically into young adult jirds. The animals are sacrificed at various post-inoculation intervals and evaluated for pathologic changes of amebic origin. The strains of amebas are also studied by electron microscopy for characteristics that might be determinants of their pathogenic index with special reference to their intracellular viruses.

Major Findings:

A number of strains of the ameba comprising formerly pathogenic strains that became non-pathogenic in axenic culture have failed to produce lesions

in the livers of jirds. These strains were all observed by EM to contain a polyhedral virus. Another strain of the ameba that contains a filamentous virus in addition to the polyhedral virus regularly produces invasive amebic lesions in the livers of inoculated jirds.

Significance to Biomedical Research and the Program of the Institute:

For nearly a century many highly skilled investigators throughout the world have sought answers to the question why morphologically identical E. histolytica often fail to evoke symptoms in some of their hosts (human) whereas in others it may kill and does so with great frequency in many areas of the world. Knowledge of factors responsible may enhance and appreciable international efforts directed toward better control and treatment of this disease.

Proposed Course of Project:

This research will be discontinued as a result of reductions in program.

Honors and Awards: None

Phillips, B. P.: Entamoeba histolytica: Concurrent irreversible loss of infectivity/pathogenicity and encystment potential after prolonged maintenance in axenic culture in vitro. Ex. Parasitol. 34: 163-167, 1973.

Diamond, L., Phillips, B. P., and Bartgis, I. L.: Virulence of axenically cultivated Entamoeba histolytica. Arch. de Investigacion Medica. 99-104, 1973.

Serial No. NIAID - 111-H
1. Microbial Immunity
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Primary amebic meningoencephalitis due to free-living amebas of the genus Naegleria

Previous Serial Number: None

Principal Investigator: Bruce P. Phillips

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	4/12			4/12
Professional:	4/12			4/12
Other:	--			--

Project Description:

Objectives:

To assess the inherent pathogenicity of this free-living species of ameba in an animal free of other microorganisms and to evaluate several potential methods of entry of the ameba into the host.

Methods Employed:

Free-living amebas of the genus Naegleria, derived from a fatal case of amebic meningoencephalitis in a 27-year-old male in Richmond, Virginia, are inoculated intranasally, intraorally, into the conjunctival sac near the inner canthus of the eyes, and into induced skin lesions in germfree guinea pigs. Animals that die or are sacrificed are examined for lesions or tissue changes in the brain, lungs, liver, spleen and intestines. Germfree isolators are carefully monitored for presence of microorganisms other than the ameba.

Major Findings:

Germfree guinea pigs inoculated intranasally with as few as 18 amebas have succumbed at post-inoculation intervals of from 6 to 11 days with amebic meningoencephalitis. None of the animals inoculated by other routes of adminis-

tration have developed symptoms and all appeared normal when sacrified up to the 84th post-inoculation day. However, the germfree animals which are a superb culture medium) have uncovered an unidentifiable diphtheroid that grows as an intracellular symbiont in this strain of Naegleria; this symbiont was not detectable by previously employed bacteriological procedures. Drug sensitivity studies of the diphtheroid have indicated its sensitivity to ampicillin and lincomycin and after six months treatment with a combination of the two antibiotics, it is hoped that the ameba have been freed of this symbiont.

Significance to Biomedical Research and the Program of the Institute:

There have been at least 57 fatal cases of primary amebic meningoencephalitis, attributable to this free-living ameba, reported from countries as remotely apart as U.S.A. (Virginia and Florida), Czechoslovakia, Australia, and England. All cases were rapidly fatal, unresponsive to drugs and all were believed to have been acquired while swimming. The organism has again been shown experimentally in the present studies to be capable of penetrating the nasal mucosa and reaching the brain. This appears almost surely to be its method of entering the host. This ameba is a very ubiquitous organism that inhabits fresh water ponds, lakes, streams, and even moist soil throughout the world. It is insensitive to 10 p.p.m. chlorine and has been isolated from chlorine-treated swimming pools and even from treated tap-water. It remains unknown why this previously harmless ameba has within the past few years become a dreaded pathogen (some strains). There is speculation that this may be a new strain, or that it may be a case of evolutionary transformation in which case it presents a potential public health problem of magnanimous magnitude. It is imperative that as much information as possible be obtained as rapidly as possible regarding this ameba and the invariably fatal disease it produces. It is not inconceivable that epidemics of appreciable proportions could erupt in any country of the world at any time.

Proposed Course of Project:

This research will be discontinued as the result of reductions in program.

Honors and Awards: None

Publications:

Phillips, B. P.: Naegleria: Another pathogenic ameba. Am. J. Trop. Med. and Hyg. In press (Sep 1974 issue). (Presented at annual meeting, Houston 1974)

Serial No. NIAID - 113
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The genetic, cellular and regulatory factors in autoallergic disease

Previous Serial Number: Same

Principal Investigator: Philip R. B. McMaster

Other Investigators: Richard Asofsky, LMI, NIAID
Michael Kyriakos*
James D. Owens, LMI, NIAID
Vernon G. Wong**

Cooperating Units: *Department of Pathology, Barnes Hospital, St. Louis, Missouri
**Department of Ophthalmology, Georgetown University, Washington, D.C.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	17/12			17/12
Professional:	6/12			6/12
Other:	11/12			11/12

Project Description:

Project Subtitle I: Genetic regulation of autoimmune ureitis

Objectives:

To elucidate the factors controlling the pathogenic autoimmune disease.

Methods Employed:

Guinea pigs were immunized with guinea pig thyroid extract and Freund's complete adjuvant, or with guinea pig retina-uveal extract in adjuvant. In certain instances, bovine gamma globulin was added to the tissue antigen mixture. The animals were observed for six weeks, then skin tested, to measure delayed hypersensitivity; their sera tested for humoral antibody and their tissues examined histologically.

Major Findings:

When immunized with ocular antigen, Hartley strain and NIH strain guinea pigs develop a severe uveitis, and Strain 13 guinea pigs develop moderate uveitis. However, Strain 2 guinea pigs do not develop any uveitis at all. All produce delayed type skin hypersensitivity and all but the Strain 2 antibody to the ocular antigen. By contrast, if immunized with thyroid antigen, the Strain 2 develops thyroiditis almost as strongly and as readily as the Strain 13.

Significance to Biomedical Research and the Program of the Institute:

The results indicate that the factors controlling the production of autoimmune disease in guinea pigs differ depending upon the organ target antigen. Elucidation of these particular differences may clarify the factors that cause the disease and lead to a better understanding of areas which can be controlled so as to treat these diseases.

Proposed Course of Project:

To investigate the types of leukocytes involved in the production of these diseases and the role of inflammatory mediators in their pathogenesis.

Project Subtitle II: The role of complement in the production of autoimmune disease

Objectives:

To determine if complement is important in the production of autoimmune nephritis, thyroiditis and uveitis.

Methods Employed:

Guinea pigs are immunized with kidney extract, thyroid extract or retina-uveal extract in Freund's adjuvant. They are examined at suitable intervals, and at the end of the study, their tissue frozen or fixed for histologic examination with ordinary light microscope or with fluorescent staining techniques for localization of antibody and complement.

Major Findings:

Guinea pigs deficient in the fourth component of complement develop as much nephritis, thyroiditis, or uveitis as others which have normal amounts of the fourth complement component following active immunization. Following passive immunization with antikidney sera, they also develop as much kidney disease as the normals.

Significance to Biomedical Research and the Program of the Institute:

This study shows that the fourth component of the complement system is not required for the production of these diseases.

Proposed Course of Project:

To study the role of the third component of complement, the production of these diseases using C4 deficient guinea pigs whose third component has been depleted by cobra venom factor.

Honors and Awards: None

Publications:

McMaster, P. R. B., Kyriakos, M., and Owens, J. D.: A comparison of autoimmunity and experiemental allergic thyroiditis in Strain 2 and Hartley strain guinea pigs. In press.

Rudofsky, U. H., McMaster, P. R. B., Wai-Sa, M., Steblay, R. W., Pollara, B.: Experimental autoimmune renal cortical tubulointerstitial disease in guinea pigs lacking the fourth component of complement (C4). J. Immunol. 112: 1387-1393, 1974.

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Serial No. NIAID - 113-E
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Immunologic Tolerance

Previous Serial Number: Same

Principal Investigator: Philip R. B. McMaster

Other Investigators: Richard Asofsky, LMI, NIAID
James D. Owens, LMI, NIAID
William Paul*
Donald Mosier*
John Finerty*****
Charles W. Boone**
Radoslav Bachvaroff***
Felix Rapaport***
Robert Weichbrodt****

Cooperating Units: *Laboratory of Immunology, NIAID, NIH
**Viral Biology Branch, NCI, NIH
***Department of Surgery, New York University School of Medicine, New York, New York
****Student
*****Work Assignment: Trudeau Institute, Saranac Lake, New York

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	17/12			17/12
Professional:	6/12			6/12
Other:	11/12			11/12

Project Description:

Project Subtitle I: The production of immunity and tolerance to pneumococcal polysaccharide when soluble, coupled to proteins and when presented as an insoluble cross linked product

Objectives:

To determine the importance of the physical form and size of immunogens and tolerogens in the induction of immunity and tolerance.

Methods Employed:

Type III pneumococcal polysaccharide is coupled to protein with nitrobenzyl bromide or cyanuric chloride, or cross linked in an insoluble sheet with formalin. These are injected in mice and guinea pigs with or without adjuvants; the response is measured with agglutinin tests or by radioimmunoassay.

Major Findings:

Large quantities of polysaccharide inoculated in animals prevent subsequent immunization with the antigen. This occurs with the same minimal dose whether the antigen is soluble or is coupled to protein to produce an insoluble product. Cross linked pieces of polysaccharide, which are also insoluble, are also effective. However, when enclosed in millipore chambers, the insoluble products have no effect even if the amount enclosed is 1,000 times the minimal amount required if not enclosed in a chamber.

Significance to Biomedical Research and the Program of the Institute:

The work indicates that animals become tolerant to these large complex haptens as readily when the complex hapten or antigens are presented as insoluable material as when presented as soluble products. The data indicate that the leukocytes need access to the insoluble antigen depot to become tolerant.

Proposed Course of Project:

To evaluate the role of the size of the antigen in the induction of tolerance and its persistence in the animal as a requisite for maintenance of tolerance.

Project Subtitle II: Mechanisms in the induction of tolerance to haptens in vitro and in vivo

Objectives:

To study the mechanisms of tolerance to a hapten, dinitrophenol coupled to T cell dependent carrier proteins, and a B cell stimulating carrier - Ficoll.

Methods Employed:

Tissue cultures of mouse spleen are prepared using spleen cells from prepared mice that had been given four days earlier 2×10^6 sheep red blood cells (SRBC) I.V. The cultures are stimulated to make antibody to dinitrophenol (DNP) by adding to the culture SRBC coupled to trinitrophenol (TNP). DNP-Ficoll derivatives are added for selected times and then washed off. The cultures are assayed for antibody forming cells using the plaquing technique. The number of cells per culture making antibody to SRBC and to DNP are compared.

Mice are injected IV or IP with the DNP-Ficoll and their serum antibody measured by the Farr test and spleen plaquing antibody forming cells measured.

Major Findings:

Adding DNP-Ficoll to a tissue culture will induce tolerance to DNP. That is, it prevents the development of antibody forming cells capable of making antibody to DNP. This occurred when DNP-Ficoll is added at the beginning of the culture or after the culture has started making antibody to DNP. If added and then washed off the cells, DNP-Ficoll still induces tolerance. The exposure time needs to last more than two hours, when the substitution ratio was less than 50 DNP groups per Ficoll molecule.

The number of DNP groups per Ficoll molecule can vary greatly and still induce tolerance. Preparations with an average of 1.2 DNP molecules per Ficoll carrier are effective and so are those with 20 or 30, even though those with a low substitution ratio are not immunogens. The amount of hapten, not the number of carrier molecules with hapten, was the most important factor. These DNP-Ficoll preparations did not alter the response to sheep RBC in vitro indicating the response was specific.

Significance to Biomedical Research and the Program of the Institute:

The system will permit an analysis of the biochemical and cellular events that are important in the induction of tolerance in vitro.

Proposed Course of the Project:

To study the importance of cell interactions and chemical mediators in the induction of tolerance with this system.

Project Subtitle III: Immunity and tolerance in vivo to dinitrophenol coupled to B cell stimulating carriers and to T cells stimulating carriers

Objectives:

To study the induction of tolerance as measured by antibody formation and skin allergy to a hapten dinitrophenol (DNP) in vivo; to delineate the mechanism of tolerance and the influence of the number of haptens per molecule.

Methods Employed:

Dinitrophenyl-ε-aminolysine is coupled to Ficoll with cyanuric chloride. Dinitrofluoro benzine was also coupled to keyhole limpet hemocyanin. These molecules were injected into mice and guinea pigs either as a solution or in Freund's adjuvants. The antibody titer was measured by radioimmunoassay and the allergic response measured by injecting the material in the foot pad, allowing one to measure the permeability change with labelled protein or by injecting the antigenic substance in the skin to observe the response. Histologic sections of the skin response were examined.

In mice, an average of 1.2 molecules of DNP per Ficoll molecule proved to be tolerogenic but not antigenic. Higher substitution ratios in mice were both

antigenic and tolerogenic in that they reduced the antibody titer to very low levels. When mice were immunized with DNP KLH and tested in the foot pad with DNP Ficoll, they responded with the permeability changes and swelling, thought associated with delayed type allergy.

In guinea pigs, an average of 1.4 DNP groups per molecule were tolerogenic but not immunogenic. Ficoll molecules with more DNP per molecule were both. In guinea pigs immunized with DNP KLH, DNP Ficoll could elicit immediate skin reactions. It also elicitied an erythematous indurated reaction that began at about 4-6.8 hours, and lasted several days, and which was characteristic in appearance of a reaction of the classical delayed type. On histologic examination, there were many mononuclear leukocytes about the blood vessels characteristic of the delayed response. However, there were also some basophils which, if prevalent, are considered to be associated with serum mediated delayed in time reactions. The reactions could be elicited with DNP 1.2 Ficoll as well as with molecules with a higher substitution ratio. None of these reactions appeared in tolerized animals.

The prolonged skin reaction was absent in animals tested six weeks after immunization provided they were deviated by skin tests five days after immunization, a process that had little, if any, effect on the antibody titer.

When DNP Ficoll was added to the peritoneal exudate cells of animals immunized with DNP KLH, it inhibited the migration of the cells, a phenomena considered a correlate of delayed sensitivity and not of serum antibody.

Significance to Biomedical Research and the Program of the Institute:

The studies indicate that tolerance to DNP can be readily induced in vivo by injecting suitable amounts of that hapten coupled to Ficoll. They also show that the tolerance is long lasting and includes not only antibody formation but delayed type of hypersensitivity. They may also show that delayed sensitivity can be elicitied with DNP on a carbohydrate carrier that is very different from the immunizing protein carrier. The substitution ratio needed to elicit the reaction was not important. Even very low non-immunogenic ratios could do this. These data suggest that there may be a delayed type allergy to this type of hapten that is independent of the carrier.

Proposed Course of Project:

To study the induction of tolerance in vivo and to ascertain the cellular events that may be important in the induction of tolerance to antibody and compare it with events producing cellular immunity.

Publications:

Mosier D. E., Johnson, G. M., Paul, W. E., and McMaster, P. R. B.: Cellular requirements for the primary in vitro antibody response to DNP-Ficoll. Definitive report May issue J. Exp. Med.

McMaster, P. R. B., Powers, K. G., Finerty, J. F., andSchiffman, G.: Tolerance to Type III pneumococcal polysaccharide in monkey. Immunol. Commun. 2(4): 361-370, 1973.

Paul, W. E., Sharon, R., Davie, J. M., and McMaster, P. R. B.: Population changes in antigen-binding and antibody-secreting cells in the course of the immune response. Symposium "Cellular Selection and Regulation in the Immune Response". In press.

Serial No. NIAID - 116
1. Microbial Immunity
2. Experimental Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Control of immunoglobulin synthesis

Previous Serial Number: Same

Principal Investigator: Richard Asofsky

Other Investigators: Martha B. Hylton, LMI, NIAID
Vilho J. Pasanen, LMI, NIAID
Rose Lieberman, Laboratory of Immunology, NIAID
Ira Green, Laboratory of Immunology, NIAID
Ethan Shevach, Laboratory of Immunology, NIAID
Elizabeth B. Muchinski*
Michael Potter*
Alexander R. Lawton, III**
Max D. Cooper**
Carl Pierce***
Joseph M. Davie****

Cooperating Units: *Laboratory of Cell Biology, NCI
**University of Alabama School of Medicine, Birmingham, Alabama
***Department of Pathology, Harvard Medical School, Cambridge, Massachusetts
****Washington University School of Medicine, St. Louis, Missouri

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	54/12			54/12
Professional:	14/12			14/12
Other:	42/12			42/12

Project Description:

Objectives:

To elucidate the genetic, developmental, and environmental factors which govern expression of immunoglobulin and antibody synthesis with respect to levels in serum and relative rates of synthesis of various immunoglobulin classes.

Methods Employed:

Immunoglobulin levels are measured by radial immunodiffusion using agarose plates impregnated with monospecific antisera. Biosynthesis is studied by incubating tissues *in vitro* in a medium containing C-14 labeled amino acids. Media are then tested for newly synthesized proteins by means of radioimmunoelectrophoresis. Antibodies are measured by passive hemagglutination, by use of specific immunoadsorbents, by a complement-dependent bactericidal assay and by determining class-specific antibody-forming cells by localized hemolysis-in-agarose gel. Plasma cell tumors are induced by inoculation intraperitoneally of light mineral oil or pristane. Immunoglobulin synthesis is suppressed *in vivo* by inoculation from birth of appropriate antibodies to mouse immunoglobulins. Synthesis of anti-erythrocyte antibodies are suppressed *in vitro* by adding appropriate antisera to mouse immunoglobulins to mouse spleen cells in a Mishell-Dutton System. Surface immunoglobulin staining of lymphocytes is accomplished by mixing fluorescein conjugated antibodies to immunoglobulins with cells. Germfree mice are employed when a controlled environment is required.

Major Findings:

The ontogeny of immunoglobulin bearing cells was indistinguishable in germfree and conventionally reared inbred BALB/c and random-bred NIH mice. Animals were born with about 1% IgM or K positive cells; this number increased about 400 fold in the first week of life, then a further 3-fold in the next seven weeks. IgA and $\gamma 2$ positive cells appeared between two and three weeks of life regardless of germfree status; $\gamma 2$ positive cells were found invariably in germfree mice, even those not synthesizing detectable $\gamma 2$.

Sera from unimmunized germfree NIH mice contain only $7S\gamma_1$ and IgM; counterpart BALB/c mice contain traces of $\gamma 2$ and IgA (between 0.01 and 0.03 mg/ml).

Anti-IgM suppresses synthesis of all classes of antibody to sheep erythrocytes by unprimed cells *in vitro*. Anti-IgG and anti-IgA produce suppression of those specific classes only. Anti-IgM suppresses synthesis only of IgM synthesis by hyperimmune cells. The same degree of suppression by anti-IgG and anti-IgA is produced in primed as in unprimed cells.

AbL mice, bred for low responses to erythrocyte antigens, have very low immunoglobulin levels. The low immunoglobulin levels are a heritable trait which can be dissociated from the immune response to sheep erythrocytes (used for selection), and pneumococcal polysaccharide Type III.

Germfree BALB/c mice inoculated with light mineral oil or pristane develop very few plasma cell tumors in contrast to the high incidence obtained in conventional animals. Germfree BALB/c mice monocontaminated with bacteria or fed tumors in much higher incidence than do uncontaminated mice, but in lower incidence and much later than conventional mice. All of the tumors produced by monoinfected germfree mice that have been examined thus far produced IgA myeloma proteins. Mineral oil or pristane granulomas synthesized small amounts of all classes of immunoglobulins within one week of inoculation. There is an incidence of reticulum cell sarcoma and lymphoma of more than 20% in all aged (> 18 months) germfree BALB/c mice.

Certain of the lymphoid tumors are transplantable. They have been typed as "B lymphocyte type" (IgM, K+ θ^-) or "T lymphocyte type" (Ig^- θ^+). A few tumors carry no markers.

Significance to Biomedical Research and the Program of the Institute:

Mice treated from birth with anti-immunoglobulin can show complete or selective hypogammaglobulineamia. The conditions produced resemble certain forms of human hypogammaglobulineamia and may serve as models, as may AbL mice, for those disorders. Furthermore, the role of antibody in resistance to infection can be evaluated best in such animals.

Nonspecific stimulation of antibody formation by syngeneic lymphoid tumors suggests that the neoplastic cells may elaborate a product which enhances the differentiation of antibody-forming cells.

The parallel development of "B" lymphocytes in germfree and conventional mice suggests that this step in differentiation does not require antigen.

Proposed Course of Project:

Subpopulations of AbL mice with respect to capacity to respond to a variety of antigens will be defined and bred. Mice suppressed with anti-IgM from birth will be used in studies of resistance to viruses, bacteria, parasites and tumor cells. Transplantable lymphoid tumors will be examined further for capacity to mediate normal physiologic function.

Honors and Awards: None

Publications:

Herberman, R. B., Nunn, M. G., Lavrin, B. A., and Asofsky, R.: Effect of antibody to θ antigen on cell mediated immunity induced in syngeneic mice by murine sarcoma virus. J. Nat'l. Canc. Inst. 51: 1509-1512, 1973.

Cantor, H., and Asofsky, R.: Paradoxical effect of antithymocyte serum on the thymus. Nature 243: 33-40, 1973

Becker, F. F., Klein, K. M., Wolman, S. R., Asofsky, R., and Sill, S.: Characterization of primary hepatocellular carcinomas and initial transplant generations. Cancer Research 33: 3330-3338, 1973.

Serial No. NIAID - 116-J
1. Microbial Immunity
2. Experimental Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The suppressive effects of heterologous antithymocyte sera on the homograft reactions in mice

Previous Serial Number: Same

Principal Investigator: Herbert C. Morse, III

Other Investigators: Richard Asofsky, LMI, NIAID
William H. Burns*

Cooperating Units: *Laboratory of Oral Pathology, National Dental Institute

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	5/12			5/12
Professional:	4/12			4/12
Other:	1/12			1/12

Project Description:

Objectives:

To investigate the action of antithymocyte seria in suppressing graft-versus-host reactions and the capacity to respond to viral infections.

Methods Employed:

Antithymocyte sera (ATS) were prepared by immunization of rabbits with two intravenous injections of BALB/c thymus cells. Graft-versus-host assays were performed by inoculating spleen cells from mice into newborn allogeneic recipients, and measuring the resulting splenomegaly nine days later. The activity of cells from animals receiving a single dose of ATS was measured with a GVH assay 2, 11, and 30 days after treatment. In addition, spleen cells from ATS-treated mice were labelled with chromium-51 and injected into lethally irradiated syngeneic recipients. Twenty-four hours lazer the mice were sacrificed and tissues removed for counting in an assay for the effects of ATS on cells migrating to various organs--spleen, peripheral lymph node (PLN), mesenteric lymph node (MLN), liver, lung and thymus.

One-month-old F_1 mice were thymectomized. One month later they were lethally irradiated and inoculated intravenously with syngeneic bone marrow cells. Seven and fourteen days later the mice were injected subcutaneously with 0.1 ml ATS. Eleven days after the last dose of ATS, they were infected intraperitoneally with varying dilutions of high titer Hepes simplex virus. Mortality in these "TX-BM-ATS" mice was compared to that in infected normal mice.

Major Findings:

Pretreatment of donors with 0.1 ml of ATS produced an 83% reduction in the GVH reactivity of spleen cells with a gradual recovery to 64% of normal reactivity at 30 days after ATS administration. By comparison, ATS effected a 60% reduction in the capacity of cells from donor spleens to migrate to lymph nodes, either PLN or MLN, at two days after ATS but there was a rapid and complete return of this function to normal levels by day 30. Studies of the migratory capacity of cells from spleens of "nude" athymic mice in combination with evaluation of the capacity of anti-theta antiserum to release counts from cells migrating to LN demonstrated that the predominant cell type migrating to LN was thymus-derived. (See also NIAID - 118 for ATS effects on direct and synergizing GVH activity in LN.)

ATS given to TXBM mice was shown to completely suppress T-cell activities. Such mice had no reactivity of spleen or PLN cells to the mitogens conconavalin A or PHA; fluorescent staining for splenic T-cells with anti-theta showed almost complete absence of theta-bearing cells; immunization with sheep red blood cells revealed a marked depression of both direct and indirect plaque-forming cell responses. B-cell functions appeared normal as assessed by responsiveness to B-cell nitogen, LPS, and number of surface immunoglobulin positive cells in spleen by fluorescence.

Whereas few normal mice were killed by undiluted Hepes simplex, a 10^{-3} dilution of the virus pool produced greater than 50% mortality in TXBM ATS mice. Reconstitution of TXBM ATS mice with high titered antibody to Herpes significantly reduced mortality when given within 36 hours of infection. Reconstitution of TXBM ATS mice with spleen cells from normal immune mice, infected with virus ten days previously, produced significantly greater protection from mortality than did spleen cells from normal mice.

Significance to Biomedical Research and the Program of the Institute:

ATS is a potent and useful immunosuppressant which has found important clinical use. These studies have yielded further supportive evidence for the role of more than one T-cell population participating in the GVH response and indicate that the T-cell population which migrates to LN from spleen cannot be equated with that responsible for GVH activity in spleen. At least one cell component responsible for splenic GVH reactivity is much more ATS-sensitive than those cells which have LN-seeking capacity.

The frequency of serious viral infections following immunosuppression with ALS and other agents is a serious clinical problem. An understanding of the immune mechanisms responsible for resistance to such infections and their perturbation by various suppressive agents is of major importance.

Proposed Course of Project:

The capacity to differentially effect subpopulations of thymus-derived cells with ATS treatment will be examined further to characterize the immune potentials of T-cells from distinct lymphoid compartments in terms of their GVH potential--both direct and synergizing--as well as their ability to participate in humoral responses.

Honors and Awards: None

Publications:

Morse, H. C., III, and Asofsky, R.: *In vivo* effects of antithymocyte serum on the homing patterns and graft-*versus*-host reactivity of murine splenic lymphocytes. *Cell. Immunol.* 11: 19-29, 1974.

Serial No. NIAID - 117
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The immunologic response of conventional and germfree mice to blood-induced infections of Plasmodium berghei

Previous Serial Number: Same

Principal Investigator: John F. Finerty*

Other Investigators: George B. Mackaness**

Cooperating Units: *Working assignment: Trudeau Institute, Saranac Lake, New York
**Trudeau Institute, Saranac Lake, New York

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	12/12			12/12
Professional:	8/12			8/12
Other:	4/12			4/12

Project Description:

Objectives:

Assigned to Trudeau Institute to learn techniques for the assay of cell-mediated immunity. To study and assay cell-mediated immunity in animals immunized or infected with the malarial parasite, Plasmodium berghei.

Methods Employed:

P. berghei parasites were removed from their erythrocytes and killed by formaldehyde treatment. The killed parasites were injected in various dosages intravenously (IV) or subcutaneously (SC) into the left hind footpad (LHFP), without adjuvants. Mice were tested for delayed sensitivity reactions (DH) by the S.C. injection of killed parasites into the right hind footpad (RFP). RFP swelling, an indication of DH, was measured 20 hours after antigen injection with a set of calipers. Non-immunized mice, used as normal controls, received antigen only, in the RFP.

Major Findings:

All mice showed evidence of DH reactions by day 4, after immunization, regardless of the route of immunization. Thereafter, DH reactions declined in intensity, but disappeared completely in mice immunized IV. However, animals immunized S.C. always demonstrated significant DH reactions throughout the period of observation.

In addition, mice immunized S.C. with malarial antigen, when given another S.C. injection of antigen, demonstrated higher DH reactions then mice given one injection and maintained higher DH than the latter. But mice immunized I.V. could not be stimulated to produce DH once they became DH negative.

Significance to Biomedical Research and the Program of the Institute:

The principal investigator has learned techniques used for assaying cell-mediated immunity (CMI) pioneered at the Trudeau Institute. This knowledge has been successfully applied to the study of CMI reactions, induced by the immunization of mice with malarial antigens, a phenomenon hitherto unreported.

Proposed Course of Project:

The next course of studies will involve the correlation of DH reactions and protection against a challenge dose of live malarial parasites.

Honors and Awards:

Invited to give seminar at the State University of New York, May 13. Title: "Cell-mediated immunity in malaria and trypanosomiasis".

Publications:

Finerty, J. F., Hyde, C. L., and Evans, C. B.: Plasmodium berghei and Erythrozoon coccoides: Antibody and immunoglobulin synthesis in germfree and conventional mice simultaneously infected. Exp. Parasitology 34: 76-84, 1973.

Serial No. NIAID - 117-E
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The immunologic response of animals immunized with trypanosomes

Previous Serial Number: Same

Principal Investigator: John F. Finerty*

Other Investigators: George B. Mackaness**

Cooperating Units: *Working assignment: Trudeau Institute, Saranac Lake, New York
**Trudeau Institute, Saranac Lake, New York

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	6/12			6/12
Professional:	4/12			4/12
Other:	2/12			2/12

Project Description:

Objectives:

Assigned to Trudeau Institute to learn techniques for assaying cell-mediated immunity (CMI). To study and assay cell-mediated immunity in animals immunized or infected with Trypanasoma rhodesiense.

Methods Employed:

Mice were immunized with killed T. rhodesiense by the injection of various dosages of antigen intravenously (IV) or subcutaneously (SC) in the axilla region or into the left hind footpad (FP). At various time intervals after immunization, mice were tested for delayed-hypersensitivity reactions (DH) by the injection of antigen into the right hind footpad. Footpad swelling was measured 20 hours later by a set of calipers. (See NIAID 117)

Major Findings:

All immunized animals demonstrated maximum DH reactions on day 4. However, IV immunized animals lost a-l evidence of DH reactions by day 14 and DH could not be elicited by further antigen injections. In contrast, SC or FP

immunized animals always showed constant DH reactions, albeit lower than those observed at day 4. Furthermore, higher DH reactions were elicited in these mice upon subsequent injections of trypanosomal antigen.

Significance to Biomedical Research and the Program of the Institute:

The ability to assay CMI in animals immunized against T. rhodesiense is important since our previous studies have shown CMI to be necessary for protection against trypanosomiasis. The principal investigator has learned techniques for assaying CMI developed at the Trudeau Institute. The data demonstrate that SC immunization induces CMI as manifested by increased DH reactions over mice immunized by the IV route. These studies will be essential in immunizing animals in a prescribed manner, so as to protect them against trypanosomiasis.

Proposed Course of Project:

The next course of studies will involve the immunization of animals with trypanosomal antigens and correlating DH reactions with protection against challenge with live organisms.

Honors and Awards: None

Publications: None

Serial No. NIAID - 118
1. Microbial Immunity
2. Experimental Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Synergy in the graft-versus-host response

Previous Serial Number: Same

Principal Investigator: Herbert C. Morse, III

Other Investigators: Richard Asofsky, LMI, NIAID
Richard Gershon*
Robert E. Tigelaar**
Michael Harrison***
Susan S. Cross****
Wallace P. Rowe, LVD, NIAID

Cooperating Units: *Department of Pathology, Yale University, New Haven, Connecticut
**Working assignment, University College, London, England
***Laboratory of Immunology, NIAID
****Microbiologic Association, Inc., Bethesda, Maryland

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	7/12			7/12
Professional:	6/12			6/12
Other:	1/12			1/12

Project Description:

Objectives:

To characterize subpopulations of lymphoid cells which interact in the graft-versus-host (GVH) reaction and to elucidate the nature of this interaction.

Methods Employed:

GVH reactions were produced by inoculation of mouse lymphoid cells from tissues such as spleen, lymph nodes (LN), thymus and peripheral blood (PBL) or combinations of such cells into newborn allogeneic or F_1 recipients. The degree of GVH activity was assayed by two methods. The Simonsen spleen weight assay, in which the reactivity of the injected lymphoid cells is measured by the degree of spleen enlargement in recipient mice was routinely used. A death assay, in

which the cumulative mortality of sublethally irradiated recipients of lymphoid cells was expressed as a function both of time and of inoculum size was also employed. Some donor mice were pretreated subcutaneously with a single dose of antithymocyte serum and cells from various lymphoid tissues were examined thereafter for their GVH reactivity.

Major Findings:

Studies utilizing the spleen weight assay have indicated that optimal expression of GVH reactions may involve interaction among two or more cell types. These populations have the following characteristics: (1) One population is present in high concentrations in tissues which are rich in recirculating lymphocytes, such as PBL or LN. The second is a population of relatively more sessile cells found in higher concentrations in thymus and spleen. (2) Both populations must have the capacity to recognize foreign antigens in the recipient. (3) Both of the cell types involved are "thymic-dependent." (4) The cell type present in excess in thymocyte suspensions appears to act as a precursor of cells which inflect immunologic injury while the cell present in excess in PBL or LN amplifies the activity of the precursor cells.

A series of experiments were designed to determine if these subpopulations of thymus derived cells represent different stages in the maturation of a single cell line or if they indicate the development of two independent T-cell lines. PLN from mice treated with ATS 2, 11, or 30 days prior to assay (see NIAID 116-J) were tested for their direct reactivity in the spleen weight assay as well as for their capacity to produce synergy when combined with normal thymocytes. Direct reactivity of PLN cells obtained two days after ATS treatment was markedly suppressed (>80%) with recovery to less than 50% of normal activity by day 30. By comparison, the capacity of such cells to produce synergy while being greatly depressed at day 2 was supranormal at days 11 and 30. The possibility that the source of amplifier cells, appearing without the prior development of precursor cells, could be migrating cells from the thymus was also investigated. PLN cells from adult mice thymectomized 2½ months prior to inoculation with ATS showed no direct reactivity in the spleen weight assay but still had the capacity to produce synergy. Spleen cells 11 days after ATS have little GVH reactivity and would also be an unlikely source for migrating GVH-active cells (see NIAID 116-J). These studies suggest that amplifier and precursor cell activities are medicated by separately differentiating lines of thymus-derived cells.

During the course of these studies, the rearing conditions used for BALB/C and F_1 mice employed in these studies were altered. Mice were taken from conventional rearing conditions to specific pathogen free conditions by Caesarian section and foster nursing on germ free mothers. Mice from the specific pathogen free colony were found to differ markedly from their predecessor conventional mice in terms of GVH activity of their lymphoid tissues. Thymus, spleen and PLN cells showed greatly increased activity on a cell/cell basis. The potential contributions of environmental factors and foster nursing on these changes in reactivity is being examined.

Other investigators have demonstrated that the capacity of parental bone marrow cells to produce GVH mortality in reciprocal F_1 recipients is reduced

when donor cells are of maternal origin. This problem was re-evaluated in studies using mixed lymphocyte reactions and spleen weight and death GVH assays as measures of parental reactivity to cells from reciprocal F_1 hybrids. Cells from either hybrid served as equally potent stimulators for both parents in the mixed lymphocyte reaction. Spleen weight GVH studies revealed that greater splenomegaly was obtained if (C57BL/6 X BALB/C)F_1's rather than (BALB/C X C57BL/6) F_1's were recipients of spleen cells from either parent. By comparison, mortality assays demonstrated that there was a striking reduction in mortality when donor cells were from the maternal strain of the hybrid cross; i.e., BALB/C spleen cells produced much less mortality in (BALB/C X C57BL/6) F_1's than did C57BL/6 spleen cells. In that reciprocal hybrid mice should be genetically identical, it is apparent that important factors unrelated to genetic endowment play a major rol in GVH reactions.

Additional studies have evaluated the effect of a mouse herpes virus infection on the GVH reactivity of lymphoid tissues from infected mice. Mouse thymic agent causes subtotal necrosis of the mouse thymus when administered within 72 hours of birth. Necrosis maximal at days 10-14 after infection and histologic recovery is complete by week 6. GVH reactivity of thymus, spleen and PLN from mice infected seven weeks previously was compared to the activity of tissues from normal mice. Reactivity curves for thymocytes and spleen cells were significantly depressed while PLN activity was essentially normal. The capacity of PLN and thymocytes from infected mice to produce synergy when combined with cells from normal tissues is being investigated.

Significance to Biomedical Research and the Program of the Institute:

The present studies have shown that different populations of thymus-derived lymphoid cells can interact synergistically in producing GVH reactions quantitated either by mortality or by splenomegaly. This points to a previously unrecognized functional heterogeneity within the peripheral pool of cells involved in cellular immune reactions against foreign tissues. Further delineation of the nature of this interaction and the characterization of the cells involved is obviously necessary for a clearer understanding of this type of immune response. In addition elucidation of the non-genetic factors influencing GVH reactions is important for our understanding of agents modulating the interaction of these cell populations. Appropriate adjustments of the cell populations involved in cell-mediated immune responses against tumor antigens might enhance the process of tumor rejection of either syngeneic or autochthonous tumors. Similarly, the elimination of small subpopulations of lymphocytes necessary for full expression of normal GVH reactivity may have significance in dealing with the problem of suppression of homograft rejection, and the the graft-versus-host reactions which complicate bone marrow transplantation.

Proposed Course of Project:

To further define the subpopulations of thymus-derived cells through differential effects of ATS treatment, migration (see NIAID - 116-J) and thymectomy on their physiologic properties. To determine the effects of viral infections on the interacting populations.

Publications:

Morse, H. C., III, Harrison, M. R., and Asofsky, R.: Graft-versus-host reactions in reciprocal hybrid mice. I. Dissociation of T-cell activities in the mixed lymphocyte reaction and two graft-versus-host assays. J. Exp. Med. 139: 721-731, 1974.

Serial No. NIAID - 118B
1. Microbial Immunity
2. Experimental Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Functional activities of subpopulations of thymus-derived lymphocytes

Previous Serial Number: None

Principal Investigator: Robert E. Tigelaar

Other Investigators: Richard Asofsky, LMI, NIAID
H. C. Morse, LMI, NIAID
Charles B. Evans, LMI, NIAID
R. M. Gorczynski*
Marc Feldmann*

Cooperating Units: *Tumor Biology Unit, University College, London, England

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	18/12			18/12
Professional:	12/12			12/12
Other:	6/12			6/12

Project Description:

Objectives:

To characterize subpopulations of thymus-derived lymphocytes involved in cell-mediated immune reactions and to elucidate the relationship(s) of these subpopulations to each other.

Methods Employed:

The cell-mediated immune response of various populations of mouse lymphoid cells after either *in vivo* or *in vitro* exposure to specific antigens is characterized by the development of activated thymus-derived (T) lymphocytes specifically reactive toward those antigens. This T cell activation can be measured in a variety of assay systems. Two such assay systems employed have been the capacity of such activated cells to (1) specifically lyse homologous antigen-bearing cells *in vitro* in a short term ^{51}Cr-release assay, and (2) to inhibit the *in vitro* migration of normal macrophages in the presence of specific antigen. Various procedures were used to attempt a physical separation of the cells responsible for both initiating and effecting these reactions;

these procedures include velocity sedimentation, equibrium density centrifugation, migration in irradiated secondary recipients, and overnight in vitro incubation. The sensitivites of these functional activities to in vitro exposure to a variety of agents with affinity for cell membrane components (such as anti-thymocyte serum, anti-theta, anti-TL, anti-Ly, cortisone acetate, antigen-antibody complexes) have also been used to further characterize the populations of cells which are involved in these reactions.

Major Findings:

Recent studies have detailed the phenomena of synergy among subpopulations of parental strain T cells in initiating the graft-versus-host (GVH) reaction in neonatal F_1 recipients. Mixtures of thymocytes and small numbers of peripheral lymph node (PLN) cells regularly give reactions significantly greater than would be expected by summation of the reactivities of the components of the mixture measured separately. (See also project report NIAID-118.)

An apparently similar type of synergy has been demonstrated in another cell-mediated immune response, the in vitro generation of lymphocytes cytotoxic to allogeneic cells. Various number of thymocytes or PLN cells or mixtures of these populations were sensitized in vitro for five days against irradiated target cells; these cells were then tested in a short term ^{51}Cr-release assay for their capacity to lyse radio-labelled target cells. Mixtures of thymocytes and very small numbers of PLN cells gave rise to cytotoxic activity far in excess of that calculated by summing the activities generated by either population sensitized alone. This synergistic cytotoxic activity was not seen with mixtures of thymocytes and PLN cells when either population was exposed either to 1500 R or to anti-theta serum and complement prior to sensitization. As in the synergistic GVH reactions, both thymocytes and PLN cells in the mixture had to have the capacity to recognize foreign antigens on the sensitizing cell; substitution of either thymocytes or PLN cells in the mixture with similar cells from mice syngeneic to the irradiated sensitizing population did not result in the generation of synergistic levels of cytotoxic activity. These results suggest that optimal generation of cytotoxic T lymphocytes may involve an interaction among subpopulations of alloantigen-reactive T cells.

An investigation of some of the physical and biological properties of the cells involved in mediating either cytotoxicity or inhibition of macrophage migration (MIF activity) has indicated that these reactions are indeed mediated by T cells and that there are at least three experimental situations in which these activities are to a large degree dissociable, suggesting that different cells mediate these particular reactions. C57BL mice were immunized to a DBA/2 tumor allograft approximately 14 days before testing for cytotoxicity and MIF activities. The tissue distribution of these activities were distinctive; both activities were present in spleens from intraperitoneally (i.p.) immunized mice, but only MIF activities was found in the PLN cells or such mice. Adoptive transfer of immune spleens cells into irradiated syngeneic recipients revealed that while a substantial amount of MIF activity could subsequently be found in PLN cells, cytotoxic activity was found predominantly in the spleens of these adoptive hosts. Velocity sedimentation analysis (which separates cells principally on the basis of size) of immune cells immediately prior to testing indicated that while the majority of cytotoxic activity was

associated with small and medium lymphocytes, the vast majority of MIF activity was associated with medium and large lymphocytes. In an attempt to separate potentially different precursor cells for these particular effector functions, normal spleen cells were fractionated by velocity sedimentation immediately prior to in vitro allosensitization; five days later these fractions were each assayed for both cytotoxic and MIF activities. These experiments showed that the fraction which generated the most cytotoxic activity was not the fraction which generated the most MIF activity.

Additional studies were carried out to investigate the effect of overnight in vitro incubation (in the absence of deliberately added antigen) on the cytotoxic and MIF activities of different sized populations of allogeneic-immune spleen cells. The activities of whole spleen cell suspensions were markedly enhanced in both assays after such incubation, and this increased activity was mediated by T lymphocytes.

Velocity sedimentation of immune cells prior to overnight incubation and subsequent testing revealed that small T lymphocytes, which were inactive in the MIF assay prior to incubation, had acquired significant MIF activity after incubation. This acquired activity, derived from the small lymphocyte pool, was shown by a second velocity sedimentation analysis to reside in both large and small lymphocytes. Similar results were obtained when small lymphocytes were cultured for 18 hours in the presence of colchicine; i.e., small initially MIF inactive cells, still gave rise to small MIF active cells. Thus the enhancement of MIF activity apparently did not depend on obligatory cell division or change in cell size during that incubation. The effect of in vitro incubation on MIF activity could be partially mimicked by short-term exposure to dilute concentrations of trypsin; small MIF inactive cells now exhibited significant MIF activity after 10 minutes' exposure. Finally, the effect of in vitro incubation could be blocked if the incubation was carried out in the presence of serum from the spleen cell donors but not if the incubation was performed in the presence of either hyperimmune serum or additional alloantigen. These results suggest that blocking factors, perhaps in the form of antigen-antibody complexes, exist on the surface of some small T lymphocytes taken from these animals, and that these factors can suppress T cell activity in both cytotoxicity and MIF assays.

Significance to Biomedical Research and the Program of the Institute:

The present studies have provided additional evidence for a cellular basis for the observed functional heterogeneity within the pool of thymus-derived lymphocytes, and suggest that this pool is composed of separable subpopulations, each with discrete and limited functional capabilities. The observation that appropriate mixtures of T cells in vitro can generate superadditive levels of cytotoxicity against cells bearing foreign histocompatibility antigens demonstrates that the phenomenon of interactions among subpopulations of T cells is not restricted to GVH reactions but rather may be a general mechanism by which a host mounts a more effective cell-mediated immune response to foreign antigens. More effective therapeutic manipulation of these cell-mediated immune responses should follow the acquisition of a clearer understanding of the physical and biological properties of these various T cell subpopulations, how they interact, the specific immunogenic determinants which can trigger them,

and their relative susceptibilities to the modulating influence of other cell types and/or the products of these other cells.

Proposed Course of Project:

To further investigate the relationship of various cell surface constituents of thymus-derived cells to the physiologic properties of these cells. Attempts will be made to identify with various isologous and heterologous antisera functional subpopulations of T cells, on the basis of unique antigenic determinants which may be expressed on the membranes of these cells and which may be accessible to bending by appropriate antibodies. In vivo and in vitro methods for generating effector T cells in the absence of antibody forming cells and their precursors (B cells) will be used to assess the role of these latter cells in modulating the differentiation and/or subsequent functional activities of thymus-derived cells. Furthermore, studies are being initiated to investigate the relative contributions of B cells and T cells to the acquired resistance of mice to infection with Plasmodium beighei.

Honors and Awards: None

Publications:

Tigelaar, R. E., and Feldmann, M.: Synergy among thymocytes and peripheral lymph node cells in the in vitro generation of cytotoxic lymphocytes to alloantigens. Transplant. Proc. 5: 1711-1715, 1973.

Gorczynski, R., Kontianen, S., Mitchison, N. A., and Tigelaar, R. E.: Antigen-antibody complexes as blocking factors on the T-lymphocyte surface. In Cellular Selection and Regulation in the Immune Response, G. Edelman, ed., Raven Press. In press.

Tigelaar, R. E., and Gorczynski, R. M.: Separable populations of activated thymus-derived lymphocytes identified in two assays for cell-mediated immunity to murine tumor allografts. J. Exp. Med. In press.

Serial No. NIAID - 119A
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Quantitative and qualitative studies on the antibody response to pneumococcal polysaccharides at the cellular level

Previous Serial Number: Same

Principal Investigator: Phillip J. Baker

Other Investigators: Jeffrey M. Jones, Philip W. Stashak, and Diana F. Amsbaugh, LMI, NIAID
Benjamin Prescott*
Norman R. Reed**
William H. Burns***
Carl T. Hansen****

Cooperating Units: *Laboratory of Microbiology, NIAID
**Department of Microbiology, Montana State University, Bozeman, Montana
***Laboratory of Oral Medicine, NIDR
****Veterinary Resources Branch, DRS

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	54/12			54/12
Professional:	24/12			24/12
Other:	30/12			30/12

Project Description:

Project Subtitle I: Role of thymic-derived suppressor cells (suppressor T cells) in the development of low-dose paralysis

Objectives:

To determine whether suppressor T cells play an active role in the development of low-dose paralysis to Type III pneumococcal polysaccharide (SSS-III).

Methods Employed:

An adaptation of the technique of localized hemolysis-in-gel was used to show that prior treatment (priming) with weakly immunogenic doses of SSS-III,

reduces the capacity of mice to respond to subsequent immunization with an optimally immunogenic dose of this antigen. Priming or immunization with antigens, other than SSS-III, was employed to assess the specificity of the unresponsiveness observed. Athymic nude, as well as thymus-bearing mice treated with T cell-depleting agents, E.G., antilymphocyte serum (ALS) or lactic dehydrogenase virus (LDV), were used to affirm that functionally active T cells are required in order to induce this form of unresponsiveness.

Major Findings:

Prior treatment with a low dose of SSS-III greatly reduces the capacity of mice to respond to an optimally immunogenic dose of this SSS-III, given a few days or weeks later; such unresponsiveness (low-dose paralysis) persists for several weeks or months after priming. Low-dose paralysis was found to be antigen-specific, dependent upon the presence of T cells, and could be abrogated by T cell-depleting agents (ALS and LDV), given at the time of subsequent immunization. These findings indicate that suppressor T cells, which exert their inhibitory effects mainly by limiting the extent to which bone marrow-derived precursors of antibody-forming cells (B cells) proliferate after immunization, are activated by low doses of antigen; the presence of such cells at the time of subsequent immunization imposes additional restraints upon the capacity of B cells to proliferate, thereby causing less than normal numbers of PFC to be produced. Aside from this inhibitory effect, priming with low doses of SSS-III also interferes with the ability of ALS to increase the magnitude of the antibody response to SSS-III. Since amplifier T cells are required in order to elicit such enhancement, this suggests that supressor T cells may also inhibit the functional activities of other types of T cells.

Significance to Biomedical Research and the Program of the Institute:

The above findings illustrate that suppressor T cells play an active and decisive role in the development of an unresponsive state to some antigens. Since such cells appear to inhibit the functional activities of both B cells and T cells, they may provide a general homeostatic control mechanism for limiting the clone size of all types of lymphoid cells, and thus serve to prevent the development of lymphoproliferative disorders and autoimmune disease; the implications of such a control mechanism have been discussed in publications cited below. The antigen-specific nature of low-dose paralysis, in contrast to the non-specific immunosuppression obtained with various cytotoxic drugs, suggests that a thorough understanding of this form of unresponsiveness might enable one to prevent the occurrence of certain undesirable immune reactions, e.g., autoimmune disease, graft rejection, without compromising natural host defense mechanisms against infectious agents.

Proposed Course of Project:

These investigations will be extended to assess the role played by suppressor T-cells in the development of an unresponsive state to other antigens of biomedical significance. Further information is being sought concerning the manner in which suppressor T cells are activated during the course of an immune response.

Honors and Awards: None

Publications:

Baker, P. J., Reed, N. D., Stashak, P. W., Amsbaugh, D. F., and Prescott, B.: Regulation of the antibody response to Type III pneumococcal polysaccharide. I. The nature of regulatory cells. J. Exp. Med. 137: 1431-1441, 1973.

Baker, P. J., Stashak, P. W., Amsbaugh, D. F., and Prescott, B.: Regulation of the antibody response to Type III pneumococcal polysaccharide. II. Mode of action of thymic-derived suppressor cells. J. Immunol. 112: 404-409, 1974.

Barthold, D.R., Prescott, B., Stashak, P. W., Amsbaugh, D. F., and Baker, P. J.: Regulation of the antibody response to Type III pneumococcal polysaccharide. III. Role of regulatory T cells in the development of an IgG and IgA antibody response. J. Immunol. 112: 1042-1050, 1974.

Baker, P. J., Stashak, P. W., Amsbaugh, D. F., and Prescott, B.: Regulation of the antibody response to Type III pneumococcal polysaccharide. IV. Role of suppressor T cells in the development of low-dose paralysis. J. Immunol. In press.

Baker, P. J., Prescott, B., Stashak, P. W., and Amsbaugh, D. F.: Regulation of the antibody response to Type III pneumococcal polysaccharide by thymic-derived cell. Proceedings of the ICN-UCLA Conference on Molecular Biology, the Immune System, Academic Press, New York. In press.

Baker, P. J., Burns, W. H., Prescott, B., Stashak, P. W., and Amsbaugh, D. F.: Role of suppressor T cells in the development of low-dose paralysis to Type III pneumococcal polysaccharide. Brooke Lodge Conference on Immunological Tolerance: Mechanisms and Potential Therapeutic Applications, Academic Press, New York. In press.

Project Subtitle II: Mode of action of thymic-derived amplifier cells (amplifier T cells)

Objectives:

To obtain information on the distribution and anatomical location of amplifier T cells, and to determine how such cells act to increase the magnitude of the antibody response to SSS-III.

Methods Employed:

Mice, given an intravenous injection of Bordetella pertussis vaccine, were examined with respect to their ability to give a normal PFC response to SSS-III. The ability to ALS to increase the magnitude of the PFC response, as well as the ability of low doses of SSS-III to induce an unresponsiveness state, was also assessed in such mice. PFC producing antibody specific for SSS-III were detected by the procedure described in the preceding section of this report.

Major Findings:

Treatment with B. pertussis produced a marked lymphocytosis which appears to be due mainly to the entry into the circulation of mature lymphocytes from tissue pools, rather than the increased production of new cells. This in itself was not found to alter the capacity of mice to give a normal antibody response to SSS-III, or the ability of SSS-III to induce low-dose paralysis. However, under such circumstances, treatment with ALS at the time of immunization with SSS-III results in the expression of a normal, rather than an enhanced, antibody response. These findings suggest that significant numbers of amplifier T cells, which are required in order to obtain ALS-induced enhancement, escape inactivation by ALS, primarily because they reside in tissue sites having limited access to ALS. During the course of our studies on the genetic control of the antibody response to SSS-III (see last year's annual report), groups of mice, derived from various crosses and backcrosses, were compared with respect to the magnitude of the PFC and serum antibody produced following immunization with an optimally immunogenic dose of this antigen. Several groups of mice produced similar numbers of PFC; however, the serum antibody titer obtained were significantly higher or lower than expected in view of the numbers of PFC obtained, and these mice differed greatly in the degree of enhancement noted after treatment with ALS. This suggests that amplifier T cells may influence to a great degree the rate of antibody synthesis by B cells.

Significant to Biomedical Research and the Program of the Institute:

Aside from increasing our understanding concerning the development of a normal antibody response and its control, any information concerning how both amplifier and suppressor T cells act to regulate the magnitude of the immune response will enable one to better define the basis for several immunological disorders and diseases of man.

Proposed Course of Project:

An extremely sensitive radio-immuno assay has been developed which will be used to obtain more precise information concerning the influence of amplifier T cells and other factors in the rate of antibody synthesis by B cells.

Honors and Awards: None

Publications:

Amsbaugh, D. F., Hansen, C. T., Prescott, B., Stashak, P. W., Asofsky, R., and Baker, P. J.: Genetic control of the antibody response to Type III pneumococcal polysaccharide. II. Relationship between IgM immunoglobulin levels and the ability to give an IgM antibody response. J. Exp. Med. In press.

LI

LPD

LABORATORY OF PARASITIC DISEASES

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Laboratory of Parasitic Diseases

National Institute of Allergy and Infectious Diseases

SUMMARY - July 1, 1973 thru June 30, 1974

INTRODUCTION

Professional personnel additions involved the return of Dr. Cheever from Egypt, and the arrival of Associates Marino, Raizman, and a Guest Worker from Sweden, Dr. Bjorvatn. Departing professionals included Associate Luban and Staff Fellow Nash, as well as Dr. Pacheco, who died.

A major administrative action occurred in the past year in the Malaria Section with phasing out of the Human Volunteer Unit at the Federal Penitentiary in Atlanta and the move of the Unit on Primate Malaria from Chamblee, Georgia to Bethesda. This reorganization of NIH malaria research resulted in inevitable losses of experienced key personnel, including Dr. Collins, but it also affords a unique opportunity to reorient and redirect our approaches in malaria research. NIAID administrative support for continued NIH efforts against this important health problem are appreciated.

RESEARCH ACCOMPLISHMENTS

PARASITE BIOCHEMISTRY AND MECHANISMS OF ENERGY METABOLISM

In parasites: The methods for isolation of microfilariae of D. immitis appear equally suitable for isolating human microfilariae to be used in metabolic studies (Harlow). Dual wave length spectrophotometry was utilized for assaying tapeworm cytochromes in Prof. Ernster's laboratory in Stockholm; we look forward to further utilization of this methodology in LPD next year (Weinbach and Harlow). Use of the same instrumentation showed that lyophilized trophozoites of E. histolytica contained no cytochromes, thus corroborating previous evidence that all iron in this parasite is non-heme iron (Weinbach and Diamond). Continuing studies on E. histolytica transhydrogenase disclosed similarities to the analogous mammalian mitochondrial enzyme, although mitochondria are absent in the amoeba. Electron microscopic analysis indicates that the enzyme is associated with a membranous subcellular particle which remains to be identified (Harlow, Weinbach and Diamond). Further work on the metabolism of E. histolytica has shown that adenylate kinase, an enzyme generally found associated with mitochondrial membranes, is present on the surface of the amoeba, and probably plays an important role in transport of metabolites across the cell membrane. Other metabolic peculiarities which distinguish E. histolytica from mammalian cells include the absence of a typical glycogen synthetase, and an apparent aggregation of particulate glycogen by protein bridges, which was readily dispersed by pronase or trypsin (Takeuchi, Weinbach and Diamond).

In mammalian cells and platelets: New insights into the role of non-heme iron in electron transport and oxidative phosphorylation were obtained in collaboration with Prof. L. Ernster and Dr. B.D. Nelson, University of Stockholm. Using submitochondrial particles derived from beef heart, the effect of iron chelators on specific sites of the respiratory chain was determined by dual wave length spectrophotometry. It was found that these chelators inhibit electron transport as a result of their ability to uncouple oxidative phosphorylation. Chelate formation in mitochondria was demonstrated for the first time. Sufficient evidence now has been adduced to assign a definite role of non-heme iron proteins in oxidative phosphorylation (Weinbach).

Continued studies on the metabolism of human platelets also indicated the role of non-heme iron in energy metabolism. It was found that the basal respiration of human platelets was stimulated by iron-chelators and by bathophenanthroline, the specific reagent for iron analysis. The effects are strikingly similar to release of respiratory control by uncouplers of oxidative phosphorylation in isolated mitochondria. These findings indicate that non-heme iron components participate in respiratory chain phosphorylations in human platelets, presumably at the mitochondrial locus (Weinbach and Muenzer).

AMEBIASIS

Amebal virus story complicated: The biologic significance of the viruses associated with E. histolytica is still not clear. All strains of axenically cultivated amebae examined (10) contain the polyhedral virus and the filamentous virus was found in two. Even a freshly isolated strain that was not yet axenized had virus. The finding that all of 43 clones derived by microisolation were found infected with virus, plus associated data, point to a lysogenic relationship between the polyhedral virus and ameba. Variations in host range between different viral isolates and strains of amebae suggest the existence of multiple virus types (Diamond and Mattern). No consistent relationship has yet been found between the amebal virus and ability of axenically cultivated amebae to produce liver abscess in hamsters. Furthermore, multiple clones of one strain of amebae vary in production of liver abscess. A historically different type of liver abscess was found to occur in the clawed jird as compared to hamster after inoculation with E. histolytica (Marino, Phillips and Diamond).

Amebic disease in India: All of twenty isolates of E. histolytica in India showed characteristics of "pathogenic" amebae and none were Laredo-type. EM study of liver biopsies from patients with amebic hepatitis suggest that abnormalities of mitochondria and endoplasmic reticulum may be present in the absence of pathology by light microscopy. Further evaluation of the Histolyticin skin test has been carried out; false positive responses that occur with increasing frequency to doses >1 µg and the failure of uniformly positive reactions in all cases of invasive amebiasis limit the usefulness of this test. (Diamond and PL 480 Indian collaborators).

AMERICAN TRYPANOSOMIASIS

More on tissue tropism of T. cruzi: Further study of liver tropism in a blood-passaged strain of T. cruzi indicated that tissue tropism was not altered by

inoculum size and muscle tropism of a vector-derived strain appeared to be a stable characteristic. Several additional blood-passaged strains are under test for possible reversal of tropism after vector-passage. Lactic dehydrogenase isoenzyme patterns in plasma of infected mice are being employed as indicators of site of tissue injury and correlate well thus far with histologic changes in heart, skeletal muscle and liver (Mercado).

Chagas' heart disease in Nicaragua: Final evaluation was completed of patients identified with known or suspected heart disease in the 1970 Nicaragua survey. Heart disease of all types was not unusually prevalent in the small sample studied (6% definite plus 19% suspected), but high prevalence of complete RBBB (8 %) and associated clinical as well as serologic features indicate presence of Chagas' heart disease in significant numbers. Although this study was limited in scope and not definitive, it demonstrated feasibility of a simple survey technique to determine community prevalence of Chagas' heart disease (Neva and Parajon).

Cell-parasite interactions: In study mechanism of action of an anti-trypanocidal drug, Bayer 2506, the doubling time of the intracellular phase of the parasite was greatly prolonged at a sustained level of 10^{-5}M as observed in the controlled-environment culture system. At somewhat greater concentrations, 10^{-4} to 10^{-5}M penetration of vertebrate cells by trypomastigotes was inhibited. Since the respective roles of parasite vs host cell during penetration or entry are so crucial, attempts are continuing to define what happens to host cell membrane in the process by ultrastructural analysis (Dvorak).

TOXOPLASMA AND RELATED ORGANISMS

Immunity to toxo in cats: Partial to complete immunity to an oocyst-producing infection by T. gondii appears to develop at the intestinal level if the initial infection has also produced oocysts. Almost all failures to challenge occurred in cats initially infected with strains of toxo that do not produce oocysts (Sheffield and Melton). Possible usefulness of a new antigen-antibody labeling test using anti-human IgG globulin coupled to horseradish peroxidase is being tested. The principle is similar to that of the indirect fluorescent antibody test but can be performed with light microscopy. Preliminary trials of the method to detect IgG antibody to toxoplasma, E. histolytica, and T. cruzi are encouraging (Lunde).

Circulating antigen in Toxo infection: Immunologic detection of circulating antigen was sought and found in the serum of mice with fatal toxo infections. While the circulating antigen must be derived from organisms it can not be accounted for by the concentration of organisms during active parasitemia. Detection of antigen in infected rabbits required concentration from the serum by affinity chromatography. Extremely sensitive tests for circulating antigen might have clinical usefulness. (Raizman and Neva).

Other coccidian-type organisms: The exact nature of the toxoplasma-like organism (WC 1170) discovered by Wallace in Hawaii remains a mystery. Infected mice initially showed sarcocysts in muscle but subsequent passage from mice to cats and back to mice now show only toxoplasma-like cysts in the muscles. Failure to find sarcocysts on later passage raises the question that initial material contained two organisms and the sarcocyst has been lost leaving an unidentified toxoplasma-like organism, WC 1170, which shows serologic cross-reactions with toxoplasma (Wallace). This organism exhibits only limited multiplication in cell-cultures (Neva) and has a unique type of storage-material by EM examination in the sporozoites which distinguishes it from T. gondii. (Sheffield). Untrastructure of Sarcoystis fusiformis, a parasite of dogs, is being studied in collaboration with Dr. Fayer at Beltsville (Sheffield).

PATHOLOGY AND IMMUNOLOGY OF MALARIA

Merozoite-red cell interaction: Analysis of factors governing red cell invasion by merozoites continues. Several observations suggest that a receptor site on the red cell wall is needed for invasion and that this can be modified to block invasion, but the chemical nature of the receptor surface remains to be determined. Under certain conditions the presence of immune serum blocks invasion not at the receptor site of the red cell but by agglutination of merozoites (Miller). Analysis of events that have been photographed in the Dvorak Chamber and reviewed by a special video-tape enlargement process has proved helpful (Dvorak-Miller).

Humoral immunity: Technical improvements in the schizont agglutination test (SAT) with P. knowlesi has permitted confirmation and extension of Brown's observations on antigenic variation of this parasite in the rhesus monkey (Powers and Miller). In seeking other methods of preparation of antigen for the IHA test in malaria the soluble antigen released from a single freeze-thaw cycle of schizonts seems superior to other procedures (Lunde and Powers). Immunization of monkeys with heat stable serum soluble antigen of P. knowlesi gave inconsistent and only partial protection. (Collins and Contacos).

Cell-mediated immunity: Blast transformation of lymphocytes by small amounts of P. falciparum antigen was demonstrated in patients variable periods of time after infection. Larger amounts of antigen appear to have a mitogenic effect in controls. Preliminary studies suggest that a lymphokine chemotactic for monocytes is released from human lymphocytes after exposure to malarial antigen. The role of the spleen with respect to monocyte chemotaxis and in relation to immunity in general is under investigation. (Wyler, Gallin and Miller).

CHEMOTHERAPY OF MALARIA

In man: Final results are now in on the efficacy of combined Clindamycin-quinine treatment against 3 drug-resistant strains of P. falciparum in 13 human volunteers. No recrudescences were observed after a 3 day course of therapy with 450 mgm of Clindamycin 4 times a day and 650 mgm quinine 3 times a day. (Miller, Wyler, Collins and Neva). The possibilities of a collabora-

tive field trial with this combined therapy for drug resistant malaria are being investigated (Powers and Miller).

In experimental animals: As an extension of previous work on the effect of the antibiotic, Clindamycin, on malaria parasites, ultrastructural changes in P. knowlesi parasites exposed to this drug were studied. The primary effect of the drug was on ribosomes of the organism whereas the effects of chloroquine, a 4-aminoquinoline, are exerted on parasite mitochondria (Powers and Aikawa). In attempts to anticipate potential problems of malaria parasites developing resistance to the antibiotics likely to be used in treatment, the likelihood of cross-resistance to minocycline and Clindamycin was tested. Although only a 6-fold increase in resistance to minocycline could be induced in P. berghei over 86 successive minocycline-treated passages, this strain was still sensitive to Clindamycin and other antimalarials (Jacobs).

PARASITE DEVELOPMENT IN THE VECTOR

Do chemosterilants affect transmission capacity? Since genetic control techniques are being examined increasingly for vector control it was important to determine whether exposure of female mosquitoes to chemosterilants might enhance or inhibit vectorial capacity for the malaria parasite. Chemosterilants and x-irradiation are the two principle methods of treating insects to be released for genetic manipulation. Although parasite development was not eliminated in sterilized female mosquitoes, it was significantly inhibited in A. albimanus. Thus, instead of exclusive release of sterilized males, sterilized females could also be released without increasing hazards of malaria transmission (Collins, Omar and Gwadz).

Infectivity of gametocytes: The variable and unpredictable results obtained in trying to infect mosquitoes that feed upon an infected patient or animal are long known to malariologists but not understood. The number of gametocytes in the blood is not the sole determining factor. The Chamblee experience suggests that splenectomy often results in enhanced mosquito infections (Collins). The capacity to carry out studies on genetic control over resistance to mosquito infection was severely crippled by absence of a reproducible and reliable system of successful mosquito infections (Gwadz). Therefore, attention was directed to the basic issue of developing a standard and reproducible system for infecting mosquitoes. This is being studied by use of an in vitro membrane feeding technique which should permit assessment of many of the factors that govern infectivity of gametocytes (Gwadz and Collins).

SCHISTOSOMIASIS

Pathophysiology: Specimens from GU surgery for complications of schistosomiasis in Egypt provided materials for study of radiologically detectable calcification in relation to egg concentration in the tissues. Calcified tissues contained 0.5 to 1.0 million eggs per gram and as few as 100,000 eggs per cm^2 may show up on a clinical X-ray. (Cheever and NAMRU collaborators). Bladder cancer was found in additional capuchin monkeys infected with S. hematobium and ureterial tumors in several gibbons infected with

the same parasite; thus, the potential for experimental models of this association has been expanded (Cheever and Kuntz of Southwest Foundation). The site of origin of the circulating antigen found in serum in heavy experimental infections has now been determined. Using the indirect fluorescent antibody technique antibody vs the circulating antigen was localized to the lining cells of the gut of the schistosome. These cells of the schistosome gut also are positive for the PASstain as would be expected from the polysaccharide nature of the antigen. Attempts to develop a radio immunoassay for detection of circulating antigen have not been successful (Nash).

Genetic work on snail vectors: Another pigmentation mutant was found in an albino stock of B. straminea. This black mantle mutant will be useful as an additional marker in genetic analysis of this vector snail. Additional abnormal growths of certain tissues of B. glabrata have been observed under conditions compatible with either genetic control or an infectious agent as the etiology. A further complication has been found in study of genetic control of vector susceptibility; namely, genetic variation in the infectivity of different strains of parasite (Richards).

FILARIASIS AND ANGIOSTRONGYLIASIS

Filarial immunology and serology: Additional results in the tests on IgE levels in cases of occult filariasis (i.e., infection, but no circulating microfilariae) confirm earlier preliminary results of a return in IgE levels toward normal after diethylcarbamazine treatment (Pacheco and Kaplan of LCI). Since a reasonably effective technique had been developed to separate microfilariae (mf) from blood with minimum contamination from other blood elements (Pacheco and Stanley), mf antigens have been prepared to study some aspects of occult filariasis. Human mf (W. bancrofti) were harvested from two Clinical Center patients from Tonga and metabolic as well as somatic mf antigens are being studied for their capacity to release histamine from basophils sensitized with IgE antibody from cases of occult filariasis (Neva, Kaplan and Pacheco). The unfortunate death of Dr. Pacheco will seriously affect filariasis research in LPD.

Studies on angiostrongylus: Search for evidence of the recently described species of Angiostrongylus costaricensis in 142 wild cotton rats of South Texas were negative (Wallace) as were a smaller number from Florida (Neva). This parasite is a close relative of A. cantonensis, the rat lung-worm which causes human eosinophilic meningitis in the Pacific and Far East. A. costaricensis was found in Costa Rica and Panama and in the former country is associated with a serious inflammatory bowel disease. Attempts have been initiated to develop a serologic test that would be useful in diagnosis of Angiostrongylus infections, but lack of specificity is still a problem (Wallace).

NEMATODE GENETICS AND NEUROBIOLOGY

Genetics: By withholding essential nutrients, such as hemin and steroid, from the growth medium of C. elegans, mutants that exhibited growth stoppage at certain molt stages were sought. By this technique hemin controlled

mutants were produced and isolated (Pertel).

Neurobiology: The fluorescent technique for histochemical mapping of the nervous system has been used with the free-living nematode, C. elegans. Norepinephrine containing neurons have been observed in the circumenteric nerve ring, and both moradrenergic and serotoninergic areas were seen in the area of genital papillae of males. It also appears that a morepinephrine-depleted mutant has been found, but this needs to be confirmed by other techniques. (Luban and Pertel).

DENGUE AND OTHER ARBOVIRUS INFECTIONS

Geography of dengue: An outbreak of dengue occurred in the Tonga Islands of the Pacific in March 1974. Although it is the same immunologic type (2) as that occurring earlier in Niue, it has not yet been featured in association with hemorrhagic or shock syndrome. Re-study of World War II sera from Panama indicated that dengue Type 3 was active in the New World at that time. Strains of dengue virus isolated directly from patient's serum into mosquitoes are being collected from different geographic areas for comparative study (Rosen).

Mosquito susceptibility to dengue: Tests of 11 different geographic strains of A. albopictus to Type 2 dengue virus showed some variation in infection rates but not to the degree expected for genetically controlled resistance. Development of virus in salivary glands of A. albopictus has been followed by the direct fluorescent technique. Infection begins in the lateral lobes of the gland with progressive involvement with time. (Gubler).

Other arbovirus infections: Several thousand human sera from South Pacific and Southeast Asian islands were surveyed for antibodies to Group A arboviruses. Antibodies to Ross River virus, the presumed cause of epidemic polyarthritis, were common in New Guinea and in several of the Solomon Islands. Distribution of 5 Group A agents in the South Pacific will be mapped by this study. The 22 viruses which comprise the Phlebotomus fever group are being subjected to a complete serologic study in collaboration with workers in Panama and at Yale for definitive classification. The susceptibility of different geographic strains of A. albopictus mosquitoes to Chikungunya virus by feeding showed considerable variation. Despite intensive inbreeding of offspring from infected or non-infected females it was not possible to select out purely resistant or susceptible lines (Tesh).

Serial No. NIAID 120
1. Parasitic Diseases
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Administration from the Office of the Chief

Previous Serial Number: Same

Principal Investigator: Franklin A. Neva

Other Investigator: Allen W. Cheever (Assistant Chief)

Cooperating Units: Section on Clinical Parasitology, Laboratory of Clinical Investigation, NIAID

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	45/12			45/12
Professional	9/12			9/12
Other	36/12			36/12

Project Description:

This project furnishes administrative and supervisory services to research projects in the Laboratory as follows:

Research plans are discussed with various investigators and overall research activities are coordinated. Travel of personnel is arranged and secretarial services are provided. Assistance is given in the preparation, editing, and reviewing of manuscripts and reports. Requisitioning and supervision of equipment and supplies and maintenance of the library is provided. Consultation and the liaison activities are maintained with individuals, academic organizations, and other branches of the Federal Government in matters pertaining to the activities of the Laboratory. Arrangements are made for supervision of research workers who are in a training status. Centralized facilities and supervision are provided for care of experimental animals used by investigators.

Liaison is maintained with the Section on Clinical Parasitology in LCI for diagnostic services or special investigations on Clinical Center patients. Dr. Theodore E. Nash had major responsibility for clinical duties in the Section.

Final stages in reorganization of the Section on Malaria are in progress. The Unit on Human Malaria at the Federal Penitentiary in Atlanta was terminated and the primate and insectary facilities at Chamblee, Georgia will be moved to Bethesda in June 1973. Unfortunately, the move from Georgia to Bethesda will result in the loss of many experienced personnel, including Dr. Collins, who have elected to stay in Atlanta with the CDC. The planning of the new insectary at Bethesda and recruitment of new personnel for the Malaria Section has required considerable time and effort, plus the much appreciated cooperation of the Scientific Director and his staff.

Other personnel changes included the entry on duty of Research Associates Joseph Marino, M.D. and Richard Raizman, M.D. in July 1973 and a Guest Worker from Sweden, Bjarne Borvatn, M.D., who will be with us until June 1974. Dr. Cheever returned to NIH after a two-year tour at NAMRU-3 in Cairo, Egypt. Dr. Omar, Ph.D., who had been with us for 3 years in Chamblee as a Visiting Fellow and Associate left in 1973 and Research Associate Norman Luban, M.D. will depart in June 1974, as will Staff Fellow, Theodore Nash, M.D. For many of us the greatest loss of all to LPD in the past year was the death of our colleague, Dr. Guillermo Pacheco, after a long illness.

The following foreign travel for research purposes by professional staff of LPD was carried out during FY 1974:

Dr. F.A. Neva to Nicaragua, Central America.
Dr. E. C. Weinbach to Sweden.
Dr. C. S. Richards to Brazil, South America.
Drs. Leon Rosen and Robert Tesh of the Pacific Research Unit to the Far East and Pacific regions.

Dr. Harley Sheffield took over as the coordinator for guest speakers and seminars. For the past two years LPD Staff has presented a series of lectures on parasitology, with particular emphasis on selected human parasites.This series was initially organized by the late Dr. Pacheco and is primarily intended as a background introduction for Associates, Staff Fellows, and Guest Workers.

Honors and Awards

F. A. Neva

Reappointment as Lecturer in Tropical Public Health at Harvard University School of Public Health, Boston, Massachusetts.

Representative of American Society of Tropical Medicine and Hygiene on the National Council for International Health, and membership on several sub-committees of this Council. Resigned as representative April 1974.

Member of Medical Advisory Board of Gorgas Memorial Institute of Tropical and Preventive Medicine, Inc.

Member of Ad Hoc Parasitology Group for Walter Reed Institute of Research for review of contract applications.

Invited participant in Macy Foundation Conference on Teaching of Tropical Medicine and Parasitology in Medical Schools in June 1974.

Invited Lectures, Conferences, or Talks Given:

1. Lecture on Chagas' disease at Harvard School of Public Health, October 1973.
2. Series of lectures on medical parasitology at University of Kentucky, Lexington in fall 1973.
3. Lecture and/or Grand Rounds at University of Pennsylvania (fall 1973); Medical College of New Jersey and United Hospitals of Neward (fall 1973); University of Pittsburgh (Feb. 1974), and Mt. Sinai Hospital of New York (April 1974).
4. Lecture at Howard University, Washington, D.C. (May 1974).

Publications:

Neva, F.A. Protozoal Diseases Endemic in the United States. Am. J. Trop. Med. & Hyg. (In Press) (Macy Foundation Symposium, New Orleans, June 1973).

Collins, W.E., Neva, F.A., Chaves-Carballo, E., Stanfill, P.S., and Richardson, B.B.: Studies on human malaria in Aotus monkeys. II. Establishment of a strain of Plasmodium falciparum from Panama. J. Parasit. 59: 609-612, 1973.

Glew, R.H., Miller, L.H., Collins, W.E., Howard, W.A., Wyler, D.J., Chaves-Carballo, E., and Neva, F.A.: Response to treatment in man of multi-drug resistant Plasmodium falciparum from Panama. Am. J. Trop. Med. & Hyg. 23: 1-7, 1974.

Publications from former LPD Staff Members:

Weinstein, P.P. and Highman, B.: Infection of the jird, Meriones unguiculatus, with the filarial worm, Dipetalonema viteae: central nervous system invasion and pathology. J. Parasit. 60: 138-149, 1974.

Oya, H. and Weinstein, P.P.: Demonstration of cobamide coenzyme in Ascaris suum. Comp. Biochem. Phy. (In Press).

Serial No. NIAID 121-C
1. Parasitic Diseases
2. Host-Parasite Relations
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The biology and control of the snail intermediate hosts of schistosomes

Previous Serial Number: Same

Principal Investigator: Charles S. Richards

Other Investigators: Dr. Margaret Kasschau, Guest Worker.

Cooperating Units: Dr. Edward H. Michelson, Department of Tropical Public Health, School of Public Health, Harvard University.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	24/12		5/12	29/12
Professional	12/12		5/12	17/12
Other	12/12			12/12

Project Description:

Objectives: To study molluscan genetics with particular attention to applications to control measures. To study prospective agents for biological control of intermediate hosts of the schistosomes. To study the biology of the snails with the aim of improving the efficiency of control measures. To study the contributions and complications of molluscan diseases and molluscan genetics to bio-medical research. To study the causes of tumors in mollusks.

Methods Employed: Selection and crossing experiments are conducted to find additional genetic markers, to characterize linkage groups, to increase knowledge of mechanisms of molluscan genetics, and to investigate further the genetic aspects of molluscan susceptibility to infection and to molluscicides.

Parasites of mollusks are studied to determine: their identity, molluscan host range, mode of transmission, localization and pathology of infection, and interrelations with schistosome infections. Electron microscopy is employed in studies on protozoa and bacteria.

Genetic studies, fresh dissections, and stained serial sections are employed to determine the causes and pathology of tumors in mollusks.

Major Findings: Established genetic stocks of B. glabrata, differing in susceptibility for infection with S. mansoni, were employed in studying the genetics of infectivity variation in different strains of S. mansoni. Results of a cross between a strain of S. mansoni from Puerto Rico with one from St. Lucia indicated that infectivity is sex-associated and that the female schistosome is XY (heterogametic), the male XX. The more infective Puerto Rican strain is essentially homogenic for infectivity, while the St. Lucia strain is heterogenic. Selection experiments are in progress to segregate from the St. Lucia strain two genetic sub-strains differing in infectivity.

A new pigmentation mutant was observed in an albino stock of B. straminea. This mutant lacks black pigment in eyes and body, but forms black mantle spots. Crosses demonstrated that this pigment type is a fourth allele of the single gene determining the other pigment types; spotted mantle being dominant over albino, but recessive to blackeye and wild type pigmentation.

Ultrastructural studies on three new species of flagellates of the genus Cryptobia, parasitizing the spermatheca of planorbid snails, revealed mitochondria, kinetoplasts, flagella, and microtubules similar to the trypanosomes, including a structure resembling the cytopharynx described in some trypanosomes.

Studies on abnormal growths in B. glabrata were continued. Cellular aggregates occur in the hemocele anterior to the digestive gland in high frequency in one clonal stock of B. glabrata. In another clonal stock the posterior lobes of the digestive gland appear to be abnormal. Frequencies of these two pathological conditions suggest either genetics or an infectious agent as the causal factor.

Hemoglobin deficiency is an inherited character in some stocks of B. glabrata. Most such snails die as juveniles. Sections of two snails that survived to maturity reveal bacterial lesions in the kidney. These appear to be the result not the cause of hemoglobin deficiency. Sections of two B. glabrata that became spontaneously detached from their shells reveal extensive bacterial invasion. Fecal impactions are not infrequently observed in the intestine of B. glabrata. This pathological condition usually leads to resorption of digestive gland and ovatestis, edema, and death.

Significance to Bio-medical Research and the Program of the Institute:

Establishing stocks of B. glabrata, of various combinations of juvenile and adult susceptibility to strains of S. mansoni differing in infectivity, provides material for bio-chemical, biological and cytogenetic intraspecific analyses of the basic mechanisms of susceptibility.

Need for evaluation of the role of genetics in snail control continues. Refractory B. glabrata may prove useful in biological control of schistosomiasis by dilution or replacement of populations of susceptible snails in endemic areas. Progress in understanding causes of susceptibility or insusceptibility of B. glabrata to infection with S. mansoni accelerates the prospect of safe field testing of refractory snail stocks for control. Development of a refractory stock of B. glabrata incompatible with the endemic snail population in a problem area may avoid dilution of the introduced insusceptibility by interbreeding.

Establishing markers for some of the various linkage groups in B. glabrata increases our capacity to analyze invisible and complex genetic characters such as host-parasite relations.

The monogenetic three allele pigment character in B. straminea provides a genetic marker enabling studies of the genetics of susceptibility in that snail species for comparison with B. glabrata.

Information (including histopathology) on a variety of tumors observed in B. glabrata, many of which are genetic, should help in analyses of tumors of mollusks collected in the field.

Microsporida, amebae, and bacteria cause extensive damage in some snail hosts. These and other snail diseases need to be examined for potential human infection, and prospective use as agents in biological control of snails. It is essential to be aware of the occurrence of these infections and their relations with the snails and other parasites to plan, conduct, and interpret experimental research on snails with validity.

Results increasingly implicate genetics in host-parasite relations involving various parasites.

Proposed Course of the Project: Genetic studies on B. glabrata and B. straminea will be continued to: (a) seek additional genetic markers: (b) expand our knowledge of linkage groups; (c) characterize and establish true breeding stocks of the various susceptibility combinations of B. glabrata and B. straminea for S. mansoni infections; (d) test additional stocks of B. glabrata and strains of S. mansoni as available and (e) continue studies of incompatibility between certain stocks of B. glabrata.

Plans have been initiated for research studies in collaboration with Dr. Frederico Barbosa, Professor of Epidemiology at the University of Brasilia. Laboratory and field trials will be carried on Brazil to assess the potential for genetic control of transmission of S. mansoni by the use of genetic stocks of B. glabrata and B. straminea refractory to infection.

Biochemical and histopathological studies will be continued, in collaboration with Dr. Kasschau (Guest worker) and Dr. T. S. Theodore (LM), to elucidate the basic mechanisms involved in variations in susceptibility of B. glabrata for infection with S. mansoni. Parabiotic methods, involving interchange of hemolymph between two snails through a connecting capillary tube, will be employed to study susceptibility for infection with S. mansoni, and intra and interspecific incompatibility. Snails will be irradiated to study effects on susceptiblity.

Studies on protozoan and bacterial infections in mollusks will be continued: including relations to the molluscan hosts, effects on other parasites, potential for human infection, and as a variable factor in experimental research.

Molluscan abnormalities will be studied by fresh dissections and culturing, serial sections, genetic selection and crossing, and environmental manipulation to determine causal factors. Studies on the histopathology of molluscan tumors will be continued in collaboration with Dr. Michelson.

Honors and Awards:

Member, editorial board, Journal of Invertebrate Pathology.

Participation in "Marine Bioassays Evaluation Workshop" sponsored by the Marine Technology Society, Montauk, Long Island, New York, April 9-11, 1973.

Publications:

Richards, C.S.: Susceptibility of adult Biomphalaria glabrata to Schistosoma mansoni infection. Am. J. Trop. Med. Hyg. 22: 748-756, 1973.

Richards, C.S.: Genetics of Biomphalaria glabrata (Gastropoda: Planorbidae). Malacol. Rev. 6: 199-202, 1973.

Richards, C.S.: Bulbous head growths of Biomphalaria glabrata: Genetic studies. J. Invert. Path. 22: 278-282, 1973.

Richards, C.S.: Tumors in the pulmonary cavity of Biomphalaria glabrata: Genetic studies. J. Invert. Path. 22: 283-389, 1973.

Richards, C.S.: Inherited variability in the course of the intestine in Biomphalaria glabrata. Malacol. Rev. 6: 133-138, 1973

Richards, C.S.: Fecal pellets of Biomphalaria glabrata. Malacol. Rev. 6: 125-131, 1973.

Richards, C.S.: Genetics of pigmentation in Biomphalaria straminea. Am. J. Trop. Med. Hyg. (in Press), 1974.

Richards, C.S.: Antler tentacles of Biomphalaria glabrata: Genetic studies. J. Invert. Path. (in Press), 1974.

Richards, C.S.: Shell mats associated with Microsporida in Biomphalaria glabrata: Genetic studies. J. Invert. Path. (in Press), 1974.

Richards, C.S.: Everted preputium and swollen tentacles in Biomphalaria glabrata: Genetic studies. J. Invert. Path. (in Press), 1974

Serial No. NIAID 121-H
1. Parasitic Diseases
2. Host-Parasite Relations
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Pathogenesis of Schistosome Infections in Mammalian Hosts

Previous Serial Number: Same

Principal Investigator: Allen W. Cheever

Other Investigators: None

Cooperating Units: Southwest Foundation for Research and Education (Drs. Kuntz and Myers); Department of Pathology, Hospital Professor Edgard Santos, Salvador, Brazil (Dr. Andrade); Department of Pathology, Peter Bent Brigham Hospital, Boston, Mass. (Dr. von Lichtenberg); Department of Zoology, University of Georgia (Dr. Damian): Division of Clinical Oncology, University of Wisconsin (Drs. Bryan and Brown).

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	22/12			22/12
Professional	10/12			10/12
Other	12/12			12/12

Project Description:

Objectives: To determine the nature and pathogenesis of the lesions produced in various mammalian hosts by schistosomes of medical importance.

Methods Employed: Physiologic parameters and lesions are related to host reactivity and quantitative parasitologic information.

Major Findings: Data from 400 autopsies performed in Cairo, Egypt

has been revised and computer analysis begun. No results beyond those previously reported are now available.

Bladder cancers have been found in an additional 4 capuchin monkeys infected with S. haematobium at the Southwest Foundation. Isologous transplants of tumors have been done and the monkeys are being followed. Urothelial tumors were also produced in 2 infected gibbons. One of these animals also developed calcification of the bladder, the first experimental reproduction of this complication of S. haematobium infection.

Pipestem fibrosis in chimpanzees infected with S. japonicum was shown to be easily prevented by early treatment but was essentially irreversible once it developed.

Significance to Biomedical Research and the Program of the Institute:

Determination of the morbidity of schistosome infections depends upon accurate information concerning the anatomic lesions and the development of criteria which allow extension of autopsy findings to living patients. The production of bladder cancers in experimentally infected primates offers an opportunity to study this tumor in a "natural" setting.

Proposed Course of the Project: Factors governing host reaction to S. japonicum eggs will be examined biologically and, if suitable collaboration is found, immunologically. Study of bladder cancers will emphasize determining the biological behavior of the tumors and changes in urinary tryptophan metabolites during schistosome infection.

Honors and awards: Dr. Cheever served on the Council of the American Society of Tropical Medicine and Hygiene and as Consultant to the WHO (Measurement of Public Health Importance of Schistosomiasis). Invited lectures were presented to graduate students in the Department of Zoology, University of Maryland and in the Department of Pathobiology, Johns Hopkins University School of Public Health. He is also a member of the TMP Study Section.

Publications:

Cheever, A.W., Erickson, D.G., Sadun, E.H. and von Lichtenberg, F. Schistosoma japonicum infection in monkeys and baboons: parasitological and pathological findings. Am. J. Trop. Med. Hyg. 23: 51-64, 1974.

Cheever, A.W., Kuntz, R.E., Myers, B.J., Moore, J.A. and Huang, T.C.: Schistosomiasis haematobia in African, hamadryas and gelada baboons. Am. J. Trop. Med. Hyg. 23: 429-448, 1974.

Smith, J.H., Torky, H., Mansour, N. and Cheever, A.W.: Studies on egg excretion and tissue egg burden in urinary schistosomiasis. Am. J. Trop. Med. Hyg. 23: 163-168, 1974.

Cheever, A.W. and Duvall, R.H.: Single and repeated infections of grivet monkeys with Schistosoma mansoni: parasitological and pathological observations over a 31-month period. Am. J. Trop. Med. Hyg. (In press)

Sadun, E.H., von Lichtenberg, F., Erickson, D.G., Cheever, A.W., Bueding, E.E. and Anderson, J.S.: The effect of treatment on the evolution of schistosomiasis japonica in chimpanzees. Am. J. Trop. Med. Hyg. (In press)

Lee, H.G.: Aspects of the effect of thioxanthone on Schistosoma mansoni in mice and in vitro. Bull. WHO 46: 397-402, 1972.

Serial No. NIAID 121-I
1. Parasitic Diseases
2. Host-Parasite Relations
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Serologic Studies on Filariasis

Previous Serial Number: Same

Principal Investigator: Guillermo Pacheco

Other Investigators: Anastasia Stanley

Cooperating Units: Dr. Thomas C. Orihel, Delta Regional Primate Center, Covington, Louisiana. Dr. Angel M. Ponce, San Pedro Macoris, Dominican Republic. Dr. Martin S. Wolfe, Department of State, Washington,D.C.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	9/12			9/12
Professional	9/12			9/12
Other	0			0

Project Description:

Objectives: To study serologic changes in experimental and natural infections with various filariae in man and animals. Antigens from different stages in the life cycle of homologous or heterologous parasites are used in these studies.

Methods Employed: Indirect tanned-cell hemagglutination (IHA) is used as the basic technic to evaluate humoral antibody response. Fluorescent antibody technics are used to demonstrate antigen-antibody complexes with soluble antigens; diffusion in agar and immunoelectrophoresis are used as adjuncts in analysis of antigens.

Major Findings: Studies of sectioned microfilariae of D. imitis using the indirect fluorescent antibody technique gave results equivalent to those obtained with microfilarial antigen in the indirect hemagglutination test. Sections of adult D. imitis are being similarly studied.

Significance to Biomedical Research and the Program of the Institute:

Serologic diagnosis of filariasis remains inadequate because of the lack of specificity of the antigens. We have attempted to evaluate currently available serodiagnostic techniques with sera from adequately studied cases and to devise more specific techniques.

Proposed Course of the Project: This project has been terminated following retirement of the principal investigator.

Serial No. NIAID 121-J
1. Parasitic Diseases
2. Host-Parasite Relationship
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Host-parasite Relationships in Filariasis

Previous Serial Number: Same

Principal Investigator: Guillermo Pacheco

Other Investigators: None

Cooperating Units: Dr. Thomas C. Orihel, Delta Regional Primate Center, Covington, Louisiana; Dr. Angel M. Ponce, San Pedro de Macoris, Dominican Republic; Dr. Allen Kaplan, LCI, NIAID; Dr. Glen Geelhoed, NCI.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	9/12			9/12
Professional	9/12			9/12
Other	0			0

Project Description:

Objectives: To study the interaction between filariae and their vertebrate hosts. These studies are directed to provide a better understanding of filariasis and other nonpatent helminthiases in man.

Methods Employed: Brugia malayi, B. pahangi, Dipetalonema viteae, Dirofilaria immitis and D. corynodes, as well as appropriate arthropod vectors, are maintained for experimental study. Blood and tissues from monkeys infected with D. corynodes or B. malayi, and people infected with Wuchereria bancrofti are received from collaborating laboratories.

Major Findings: A new technique was developed for separation of large numbers of clean microfilariae from whole blood. No new experimental infections were carried out.

Significance to Biomedical Research and the Program of the Institute:

Altered host-parasite relations are apparently basic to much of the disease produced by filarial infections. We have sought to explore altered host-parasite relations in experimental models and apply these observations to disease syndromes occuring in man.

Proposed Course of the Project: This project has been terminated following retirement of the principal investigator.

Honors and Awards:

Dr. Pacheco served as a Member of the U.S. Panel of the U.S.-Japan Cooperative Medical Science Program (Parasitic Diseases).

Serial No. NIAID 121-K
1. Parasitic Diseases
2. Host-Parasite Relationship
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Detection and characterization of a circulating schistosome antigen and other immunologic studies in schistosomiasis.

Previous Serial Number: Same

Principal Investigator: Theodore E. Nash

Other Investigators: Franklin A. Neva and Benjamin Prescott

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	6/12			6/12
Professional	6/12			6/12
Other				

Project Description:

Objectives: (1) To determine the location of the schistosome derived circulating antigen in the adult schistosome. (2) To develop a radioimmunoassay to this antigen. (3) To determine the location of the antigen in the infected host. (4) To determine whether this antigen can be used in diagnostic serologic tests for schistosomiasis.

Methods Employed: Using specific antisera to this antigen, immunofluorescent techniques were employed to determine the location of the antigen in adult schistosomes and infected animals. The cyanogen bromide technique was used in attempts to bind thyramine to the antigen followed by labelling with $_{125}$I. Anti-antigen rabbit gamma globulin was prepared and labelled with $_{125}$I. Polyvinyl plates were used in an attempt to develop a solid phase radioimmunoassay. Standard complement fixation techniques were employed

using the antigen and whole worm antigens reacting with infected patients sera. Lipid fractions of worms were separated and tested for complement fixating ability.

Major Findings: The location of the circulating antigen in the adult schistosome is in the lining cells of the gut, the border of the lining cells of the gut and the lumen of the gut, and occasionally in the lumen itself. Studies in infected mice have failed to locate the antigen in the liver. PAS staining of the schistosome revealed positive staining in areas corresponding to the fluorescence observed using specific antisera to the antigen.

Attempts to develop a radioimmunoassay by labelling the antigen have not been successful. Presently studies are in progress using a solid phase system with purified labelled anti-antigen rabbit globulin.

Studies using the antigen in complement fixation tests revealed that whole worm antigens were more sensitive and specific in diagnosing known schistosome infections than the polysaccharide antigen. Positive CF to the antigen activity was observed in patients whose sera contained precipitating antibodies to the antigen. Lipid fractions cross reacted with various bacterial and mycoplasmal lipids.

Significance to Bio-medical Research and the Program of the Institute:

The finding that the antigen originates from the gut of the schistosome is intriguing. Unraveling the role this substance plays in the physiology of the trematode can lead to new therapeutic approaches. Structural polysaccharides have not been studied in schistosomes. The antigen may play a role in the development of glomerulonephritis in humans. Development of a radioimmunoassay can lead to development of a way to quantitate human infections.

Proposed Course of Project: Termination due to depature of principal investigator.

Publication:

Nash, T.E., Prescott, B. and Neva. F.A.: The characteristics of a circulating antigen in schistosomiasis. J. Immunol. 112: 1500-1507, 1974.

Nash, T.E.: Location of the circulating antigen in schistosomiasis. Amer. J. Trop. Med. Hyg. (in Press), 1974.

Serial No. NIAID 122-C
1. Parasitic Diseases
2. Parasite Growth and Differentiation Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1974

Project Title: Studies on Entamoeba histolytica and other parasitic protozoa.

Previous Serial Number: Same

Principal Investigator: Louis S. Diamond

Other Investigators: Joseph T. Marino, Carl F. T. Mattern, and Bruce P. Phillips

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	51/12			51/12
Professional	20/12			20/12
Other	31/12			31/12

Project Description:

Objectives: To develop and refine techniques for the axenic cultivation of Entamoeba histolytica, related amebae and other parasitic protozoa; to define their in vitro nutritional requirements, to study their metabolism, and mechanisms of pathogenicity. To study naturally occurring viruses of E. histolytica. To develop techniques for cryopreservation of parasitic protozoa, to study mechanisms involved in resistance of these organisms to freezing injury.

Methods Employed: Entamoebae or other parasitic protozoa freed of naturally associated microorganisms by use of microisolation procedures and/or a variety of antibiotics are established in monoxenic cultures with single species of trypanosomatids or bacteria, then adapted to grow under axenic conditions. In vitro growth is monitored through use of a

Coulter electronic counter and/or standard counting chambers.

Viruses of E. histolytica are characterized and studied using classical virological techniques.

Protozoa are suspended in a variety of cryopreservatives and the effects of varying freezing and thawing rates on recovery of viable cells cooled to temperature of liquid nitrogen (-196°C) are studied.

Laboratory animals are inoculated via laparatomy with amebae cultivated under a variety of conditions and pathogenesis is studies.

Major Findings: During the past year the principal research efforts in this Laboratory have been concerned with 1) Elucidation of the relationships between Entamoeba histolytica and its indigenous viruses, 2) Determination if any, of the relationships between amebal virus and virulence of the amebae, 3) Histo-pathological study of hepatic amebiasis in the hamster and jird.

To-date 10 strains of E. histolytica have been examined and found infected with indigenous viruses. All of the strains were infected with a polyhedral virus. In addition, two of the strains were also infected with a filamentous virus.

A third morphologic entity having a unique beaded structure has been found in three of the above strains. Attempts are being made to isolate and determine the possible viral nature of this entity.

The amebal host range of the viruses of each amebal strain has been studied by cross-infection experiments in heterologous strains of amebae. The data indicates that each viral isolate has a different host range which in turn suggests that more than one viral entity is involved.

In studies of the host range of the viruses isolated from several clones of HB-301:NIH amebae it has been shown that one amebal strain, HK-9, is consistently more sensitive to all of these viruses than the other amebal strains. Apparently, fewer infectious doses are required to bring about CPE and complete lysis occurs more rapidly. These findings point to genetic differences between the hosts.

Indigenous virus production in both cloned and uncloned (wild type) amebal cultures varies between strains being consistently high in some and low in others. Moreover, in some clones and wild type strains virus production fluctuates from high to low to high.

Two clones of strain HB-301 amebae known to be consistently low-producers of polyhedral virus have been induced to produce relatively high titers by cultivating the amebae with BUDR for a short period. Subsequently, the high titers returned to their original low levels after the treated amebae were subcultured for several weeks in the absence of BUDR. A third

clone did not respond to similar exposure to BUDR.

Forty three clones representing 3 generations of HB-301 amebae have been examined and found infected with virus. These findings together with earlier findings that prolonged exposure of the amebae to specific viral-antiserum fails to result in freeing the amebae of its virus strengthens the concept that the polyhedral virus-ameba system is a lysogenic one.

The virulence of 9 strains of axenically cultivated E. histolytica has been evaluated by direct inoculation into the liver of the golden hamster. Based on an average lesion score (0-4) virulence among the strains varied from 0 to 3.5. Furthermore, in contrast to the findings of Wittner and Rosenbaum there appeared to be no strict correlation between degree of virulence and period of time the amebae were in axenic culture. One strain in axenic culture 12 years produced lesions in 9 of 20 animals (45%) and in two of the animals severe grade 3 lesions developed.

Clones of axenically cultivated HB-301 amebae assayed in the golden hamster via direct inoculation of the liver have been shown to vary significantly in their ability to invade and destroy hepatic tissue. Since all of these clones were isolated at the same time, ruling out the possible effect, if any, of the axenic state on virulence, these findings can be interpreted as being indicative of the existence of histotrophic clones of E. histolytica.

A comparative study of induced hepatic infection with E. histolytica in the clawed jird and golden hamster revealed noteworthy differences in the histopathology of the two species. Namely, the occurrence of discrete abscesses in the jird versus more confluent ones in the hamster. A chronic fibrous reaction in the former versus an acute cellular reaction in the latter, and the presence of large numbers of apparently normal amebae in the jird compared to the relative few in the hamster. These findings together with the fact that none of the jirds succumbed to infection whereas many of the hamsters did, suggests that the jird is more competent in containing hepatic infection.

Significance to Biomedical Research and the Program of the Institute:

The pathogenesis of hepatic amebiasis, the most important extra-intestinal form of the disease in humans is poorly defined. Why certain individuals develop these lesions is unknown. The existence of hepatotrophic strains has been postulated, but there is no evidence of this in nature. The finding that certain clones of a strain of E. histolytica are more virulent than others for the hamster liver lends credence to this hypothesis. Whether this is a stable genetic variant controlled by an integrated chromosome, by a cytoplasmic episome-like factor or by viral genetic material has yet to be determined.

As a result of the investigations on the clawed jird, parasitologists now have a second animal species with which to study hepatic amebiasis.

Study of the relationships between the viruses of the relatively simple eukaryote, E. histolytica, and the amebal host yields data of value to investigators dealing with mammalian-virus systems and phage.

See projects NIAID 123-C and 125-K for additional investigations on axenically cultivated E. histolytica.

Proposed Course of the Project: Efforts will be made to further characterize the physical and chemical properties of the viruses of Entamoeba. A classification of these viruses based on host range specificity is being developed. Avirulent strains of E. histolytica have been obtained and are in the process of being assayed for virus. The search for viruses in other species of Entamoeba as well as other pathogenic protozoa will continue. The possible role of amebal viruses in hepatic amebiasis will be investigated using the hamster and jird animal models. Patients with amebic disease are being screened serologically for antibodies against amebal viruses. Efforts to develop techniques for the axenic cultivation of other parasitic protozoa will continue, as will efforts to improve existing techniques for the cryopreservation of these organisms.

Honors and Awards:

Chairman, Advisory Committee to the Collection of Protozoa, American Type Culture Collection.

Member, Board of Trustees and Executive Council, American Type Culture Collection.

Member, WHO Expert Advisory Panel on Parasitic Diseases (General Parasitology).

Member, Editorial Board, Cryobiology.

Invited Conference Leader, Sixth Seminar on Amebiasis, sponsored by National University of Mexico and the Mexican Institute of Social Security, Mexico City, February 20 to 22, 1974.

Presided over Section 2 (Amebiasis) as Honorary Chairman, 9th International Congress on Tropical Medicine and Malaria, Athens, Greece, October 14-21, 1973.

Publications:

Hruska, J. F., Mattern, C. F. T., and Diamond, L. S.: Viruses of Entamoeba histolytica. IV. Studies on the nucleic acids of the filamentous and polyhedral viruses. J. Virol. 13: 205-210, 1974.

Mattern, C. F. T., Hruska, J. F., and Diamond, L. S.: Viruses of Entamoeba histolytica. V. Ultrastructure of the polyhedral virus V_{301}. J. Virol. 13: 247-249, 1974.

Diamond, L. S., Phillips, B. P., and Bartgis, I. L.: Virulence of axenically cultivated E. histolytica. Arch. Invest. Med. (Mex.) 4, Supl. 1: 99-104, 1973.

Diamond, L. S., Phillips, B. P., and Bartgis, I. L.: A comparison of the virulence of nine strains of axenically cultivated Entamoeba histolytica in hamster liver. (In press)

Diamond, L. S., Phillips, B. P., and Bartgis, I. L.: The clawed jird (Meriones unguiculatus) as an experimental animal for the study of hepatic amebiasis. (In press)

Weinbach, E. C. and Diamond, L. S.: Entamoeba histolytica. I. Aerobic metabolism, Exp. Parasit. 35: 232-243, 1974.

Serial No. NIAID 122-J
1. Parasitic Diseases
2. Growth and Differentiation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Biochemical Basis for Growth and Differentiation of Parasitic and Free-living Nematodes

Previous Serial Number: Same

Principal Investigator: Dr. Ruth Pertel

Other Investigators: Dr. Norman Luban and Dr. Rose Ann Cattolico

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	24/12			24/12
Professional	20/12			20/12
Other	4/12			4/12

Project Description:

Objectives: To investigate the generalized life cycle of parasitic and free-living nematodes utilizing techniques from biochemistry, genetics and neurobiology. To investigate sensory modulation in the wild type of C. elegans and its mutants at various stages of differentiation and development in an attempt to eleucidate the sensory mechanism involved in the transitional phase of nematodes when a free-living mode is exchanged for a parasitic mode and the development of the reproductive system begins.

To develop techniques of investigating neuronal network synaptic contacts, and morphogeneisis of the gonads in the large number of mutants altered in behavior and postembryonic development of the transitional phase. Other systems such as the mammalian taste bud have been used to supplement these studies.

Methods Employed: Free-living Caenorhabditis elegans var. Bristol cultured as synchronous populations under axenic conditions (free of all living associates) are used as the initial test system. Chemotactic, motility, drug resistant, and developmental mutants are used to study the neuronal basis for the responses of nematodes to their environment which controls reproduction. Developmental mutants are studied in regard to their genetics, population dynamics, and media effects upon growth and development.

Chemotactic assay: A biphasic (semisolid agar and aqueous system) is used to determine the sensitivities of various mutants to chemical gradients.

Anatomical studies: Conventional techniques of electron and light microscopy are used.

Major Findings:

1. Anatomical studies: Histochemical mapping of the nervous system has been attempted using the technique for demonstrating catecholamine fluorescence described by Falck and Hillarp. Norepinephrine containing neurons have been observed in the circumenteric nerve ring and both noradrenergic and serotoninergic areas have been observed in the area of the genital papillae of males. A norepinephrine depleted mutant has been observed and corroboration is being sought using a fluorometric assay (trihydroxyindole method) for norepinephrine. (Luban and Pertel)

2. Sensory mechanisms: The effect of combinations of cations on the chemotactic response has been pursued with inhibitors of ion channels such as tetrodotoxin. Divalent cations may have important effects on modulating responses to gradients.

3. Genetics: The holding stage (ensheathed dauerlarva) of parasitic nematodes prior to infection of the host usually occurs at some point prior to the development of the gonads. In the Strongyles and in C. elegans (a putative evolutionary relative of the Strongyles), development of the gonads begins at the third to fourth stage molt transition of the juveniles. We are interested in the degree that genetic vs. environmental information affects this transition. Deletion from the growth medium of hemin and steroid, essential nutrients of C. elegans, produced environmentally determined holding stages, i.e., growth stoppage with maintenance of a particular molt stage. Specific mutants controlled by hemin have been isolated and further characterized, in terms of growth, translational controls and genetics.

4. In situ hybridization: Germ-line DNA isolated from reproducing adults in which approximately one-half of the cells are derived from the embryonic germ line was hybridized to the germ-line cell DNA of larval stages which contain only 4 germ-line cells, in situ. This serves to mark the specific nuclear and cellular location of particular nucleic acids

as associated with the germ line. (Cattolico and Pertel)

Significance to Biomedical Research and the Program of the Institute:

Nematode infections of man, domestic animals, and plants are protean in character and world-wide in distribution. The studies on the mechanism of sensory processing of environmental information by nematodes would provide information on how nematode parasites find their hosts and specific tissues in the host, as well as providing information on sensory mechanisms such as taste in higher organisms.

The nematode system also offers a good study system for the understanding of the basic biology of differentiation and development. It is an in vivo system that uniquely separates the differentiation of one tissue, the gonaed, from other fully differentiated tissues. Most other systems for studying the differentiation of the blast stages of a tissue are in vitro cell cultures. These systems are plagued with contamination by transforming viruses which change the genetic characteristics of the cells, and by gene dosage effects when the chromosomes undergo abnormal ploidy changes. The information derived from the nematode studies will also provide understanding of the basic interactions attendant upon the development of parasitic nematodes as influenced by the host which so often occurs at the third to fourth molt stage transition when the gonad develops and the primary biological business of reproducing new parasites begins.

Proposed Course of the Project: The project will hopefully be continued along the general lines mentioned above.

Honors and Awards:

Ruth Pertel:

Invited to organize, chair and speak, Symposium on "Invertebrate Model Systems and Techniques for the Study of Aging". Gerontological Society Meetings, Miami, Florida, November 1973.

Invited Lectures on postembryonic genetics and development of a nematode at the following:

1. School of Life Sciences, University of California at Santa Cruz, February 1974.
2. Department of Nutritional Sciences, University of California at Berkeley, California, February 1974.
3. Department of Biology, University of California at San Diego, February 1974.
4. Department of Biology, California Institute of Technology, February 1974.
5. Department of Genetics, University of California

at Berkeley, February 1974.

6. Department of Biology, University of Southern California, May 1974.

Serial No. NIAID 123-B
1. Parasitic Diseases
2. Physiology and Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1974

Project Title: Pathological physiology and histochemistry of parasitic diseases

Previous Serial Number: Same

Principal Investigator: Teresa I. Mercado

Other Investigators: None

Cooperating Units: Comparative Pathology Section, Division of Research Services (Dr. Anton M. Allen)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	24/12			24/12
Professional	12/12			12/12
Other	12/12			12/12

Project Description:

Objectives: To gain a better understanding of the pathological physiology of parasitic diseases by physiological and histochemical studies.

To examine differences between strains of Trypanosoma cruzi by studying the susceptibility of host tissues to parasitic invasion and assessing the alteration of certain parameters.

Methods Employed: Tissues from mice infected with Trypanosoma cruzi strains of different origins are studied by means of standard histological procedures, staining with Giemsa, and histochemical assaying for lipid and glycogen. Parasite levels are determined by hemocytometer counts of blood. Plasma and tissue isoenzymes of lactate dehydrogenase are assayed with disc gel electrophoresis and staining with tetrazolium salts. Trypomastigotes of T. cruzi are isolated from the blood of infected mice by means of DEAE

cellulose columns and centrifugation at 4°C.

Major Findings: Studies reported last year indicated that vector-derived strains of T. cruzi exhibited a characteristic tropism for muscle but not for liver. This was in marked contrast to the distinct reticulotropism exhibited by a strain (Tulahuen) which had been maintained exclusively by blood-passage for many years. In an attempt to analyze the apparent differences in tropism the possible influence of the size of the inoculum was examined. The resulting infections indicated, however, that the qualitative aspects of the inoculum, rather than the quantitative, probably influenced the inability of the vector-derived strains to establish themselves in the liver i.e., the inoculation of metacyclic and epimastigote forms in the vector-derived strains versus trypomastigotes in the blood-passaged infections. The question whether or not multiple blood-passaging alters a strain's tropism was also examined. However, 10 consecutive blood passages of the House 510 strain did not alter its characteristic invasiveness of muscle and lack of invasiveness for liver, and even when massive inocula were administered (up to 18 million trypomastigotes) six additional passages were required before some parasitization of the liver occurred. The resulting parasitic foci however, were not observed frequently and differed from those produced by a typical reticulotropic strain. They appeared disorganized, and deteriorating parasites were enmeshed in nests of mononuclear cells. The only marked difference observed after multiple blood-passaging of the House 510 strain, was the occurrence of many large parasitic foci in the brain of mice which had survived periods of patency as long as 21 days. Blood-passaging of another strain, the House 11, did not produce parasitization of the liver, but the vector-passaged Tulahuen, which had not shown its typical liver invasiveness after one passage in Rhodnius, quickly reverted to its reticulotropism after only one blood-passage in mice.

Lactate dehydrogenase isoenzymes of plasma from mice infected with the House 510 of T. cruzi exhibited a distinct increase of fractions 1 and 2 (indicating heart muscle injury) and of fractions 4 and 5 (indicating injury to liver and skeletal muscle), as the infection progressed. Planimeter readings of zymograms, including all the isoenzyme fractions, ranged from 14 sq. cm. for uninfected mice, just before inoculation, to 70 sq. cm. for infected mice on the 15th. day of patency. Infections with the Tulahuen strain revealed an additional isoenzyme (LDH-X), slower-migrating than LDH-5 and occurring in larger concentrations than a similar fraction obtained with the House 510 strain, but in the latter LDH-4 was particularly prominent. These findings indicate the usefulness of isoenzyme studies in assessing tissue injury and suggest their potential value in the classification of clinical T. cruzi infections.

Significance to Biomedical Research and the Program of the Institute:

Basic cellular studies provide a means for understanding the physiological mechanisms involved in the response of the host to parasitic infections and may lead to the identification of specific parasite substances

involved in pathogenesis. Histochemical studies aid in localyzing the altered sites within tissues. Physiological studies quantitate the alteration of activity observed histochemically and aid in defining further the physiological mechanisms that may be damaged. Basic studies on strain differentiation aid in taxonomic classification and in assessing the pathogenicity of a parasitic infection.

Proposed Course of the Project: Studies on host pathology with different strains of T. cruzi are nearing completion and the manuscript will be submitted for publication. The isoenzyme studies will be continued employing more strains of T. cruzi and more specific isoenzyme procedures, e.g., creatine phosphokinase, for the detection of skeletal muscle injury and pyruvic-glutamic transaminase for the detection of liver injury. Studies will be initiated on the isoenzymes of T. cruzi trypomastigotes, recently isolated from the blood of mice.

Publications:

Mercado, T. I.: Plasmodum berghei: Inhibition by splenectomy of a paralyzing syndrome in infected rats. Exper. Parasit. 34: 142-147, 1973.

Serial No. NIAID 123-C
1. Parasitic Diseases
2. Physiology and Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Biochemical mechanisms of energy metabolism in mammalian and parasitic organisms.

Previous Serial Number: Same

Principal Investigator: Eugene C. Weinbach

Other Investigators: Louis H. Diamond, Dan Harlow (Staff Fellow), and Tsutomu Takeuchi (Visiting Fellow)

Cooperating Units: Laboratory of Microbiology, NIAID (Theodore S. Theodore) (Project No. 63B), Department of Biochemistry, Case Western Reserve University, School of Medicine (Joseph Muenzer), Department of Biochemistry, University of Stockholm, Sweden (Lars Ernster, B. Dean Nelson)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	35/12		12/12	47/12
Professional	21/12		12/12	33/12
Other	14/12			14/12

Project Description:

Objectives: To conduct fundamental studies on the mechanism of aerobic energy metabolism in mammalian and in parasitic organisms. Specifically: To continue studies of electron transport and oxidative phosphorylation in mammalian cells and mitochondria, and (B) comparative studies with cells and mitochondria from parasites.

Methods Employed: Animal tissues are fragmented by mechanical or enzymatic disruption of the cells and mitochondrial fractions are isolated by differential centrifugation. Submitochondrial particles are prepared from intact mitochondria by appropriate chemical or mechanical means. Similar

procedures are used to isolate mitochondria from cestodes, the principal metazoan parasites used in current studies.

Entamoeba histolytica, strain HK-9, is cultivated axenically in Diamond's liquid medium, and the active trophozoites collected from 72-hours cultures by centrifugation. For biochemical studies, the trophozoites are sonicated and the various fractions obtained after centrifugation are used in enzymatic, spectrophotometric and polarographic assays.

Human platelets are isolated from freshly drawn whole blood by differential centrifugation.

Mesosomal vesicles and plasma membranes are isolated from Staphlococcus aureus by differential and sucrose gradient centrifugation after treatment of the bacteria with lytic enzymes.

Cellular and mitochondrial constituents are determined and characterized by appropriate enzymatic, chemical and physical methods. Oxygen consumption is determined polarographically with the Clark oxygen electrode. Enzyme activities are assayed by spectrophotometric and radionuclide methods.

Major Findings:

A. Parasite studies

1. Cestode mitochondria: In collaboration with Prof. L. Ernster and Dr. B.D. Nelson, University of Stockholm, spectral studies of the mitochondria of Hymenolepis diminuta were initiated this year. Analyses of the pyridine hemochromogens with dual wavelength spectrophotometry, after differential extractions, identified cytochromes a,b, and c-c_1. Approximately 0.3 nanomoles of the cytochromes per mg protein were found in the parasite mitochondria. In these lyophilyzed preparations enzymatic reduction of the respiratory pigments did not occur, whereas chemical reduction with dithionite was readily achieved. Other aspects of this study are reported by Harlow (Serial No. 123-F).

2. Diaphorase activities in Entamoeba histolytica: Continued studies on the biochemistry of E. histolytica demonstrated unambiguously that the end-products of reduced pyridine nucleotides oxidation mediated by diaphorase are NAD and NADP. This was ascertained by specific enzymatic procedures. Spectral analysis of extracts of lyophilyzed trophozoites indicated the absence of cytochromes. This was corroborated by direct chemical and physical analyses utilizing atomic absorption spectroscopy which disclosed that all of the iron could be accounted for as non-heme iron. The large content of non-heme iron (12 natoms/mg protein), together with the sensitivity of the diaphorase reaction to iron chelators, indicates that iron-sulfur proteins serve as electron carriers in E. histolytica.

3. Adenine nucleotide metabolism in E. histolytica: Continued collaboration with Dr. T. Takeuchi (Visiting Fellow, Keio University, Japan) disclosed that adenylate kinase was on the cell surface and its catalytic site faced toward the extracellular space (use of immobilized enzymes and atractyloside). Sensitivity to atractyloside indicates presence of an adenine nucleotide translocase in the cell membrane of this parasite. Incubation of intact trophozoites with ADP and determination of phosphorylated intermediates demonstrated that adenylate kinase has an important role in metabolic regulation in E. histolytica, in addition to mediating the synthesis of ATP. It appears that the synthesis of ATP involves a transfer of phosphate from a protein complex to ATP.

4. Glycogen localization and metabolism in E. histolytica: This study was initiated in collaboration with Dr. T. Takeuchi. In contrast to mammalian tissues, most of the particulate glycogen and phosphorylase activity was concentrated in the large particle fraction (15,000 x g) of E. histolytica. Other enzymes associated with glycogen metabolism were found in both the soluble and particulate fractions. Phosphoglucomutase was found in the soluble fraction, and UDPG pyrophosphorlase was present in the soluble and particulate fractions. The distribution of the latter enzyme in these fractions was dependent on exogenous Mg, and its activity was inhibited by pyrophosphate. Sucrose density gradient centrifugations, in conjunction with specific enzymatic procedures, demonstrated that phosphorylase was particle bound. Glycogen synthetase of the mammalian type was not detected. Particulate glycogen was disbursed by pronase or trypsin. It is concluded that the organization of the particulate glycogen and the enzymes related with glycogen metabolism in E. histolytica differ from their mammalian counterparts.

5. Respiratory metabolism of Staphlococcus aureus: Studies of the oxidative activities associated with mesosomal vesicles and plasma membranes of S. aureus were continued with the collaboration of Dr. T. Theodore. The cytochromes and oxidases (terminal and comlete sequence of the electron transport chain, respectivity) were demonstrated to be localized primarily in the plasma membranes. Employing lactate, malate, succinate and glycerol-3-phosphate as respiratory substrates, the dehydrogenase activities of plasma membranes were found to be 2-9 times greater than those of the mesosomal vesicles. In terminating these studies, it is concluded that the respiratory chain of S. aureus is associated predominately with the plasma membrane fraction.

B. Mammalian Studies

1. Mitochondria: In collaboration with Prof. L. Ernster and Dr. B.D. Nelson, University of Stockholm, a study was conducted on the role of non-heme iron in electron transport and oxidative phosphorylation. Using submitochondrial particles derived from beef heart, the effect of iron chelators (thenoyltrifluoroacetone, TTFA) on Sites I and II of the respiratory chain was determined by dual wavelength spectrophotometry. The data indicated that TTFA either inhibits electron transport between cytochrome b and NAD, or

acts as an uncoupling reagent. Energization of the mitochondrial membrane by addition of ATP led to changes in the spectral characteristics of the cytochromes. Subsequent addition of TTFA inhibited reduction of cytochrome b and induced spectral changes in other respiratory carriers. It was established that Site I was more sensitive to the non-heme iron chelator than was Site II. Using known uncouplers of oxidative phosphorylation in analogous experiments, it was concluded that TTFA inhibits electron transport as a result of its uncoupling ability. Chelate formation between TTFA and $FeCl_3$ in ethanol was demonstrated by difference spectrophotometry and showed that 3 moles of TTFA chelates 1 mole of Fe. Chelate formation in mitochondria also was demonstrated for the first time. Although we had preliminary information that non-heme iron components are involved in oxidative phosphorylation, these studies provide new and cogent evidence bearing on the role of metals in energy conservation reactions of the mammalian respiratory chain.

2. Energy metabolism in human platelets: Continued studies on the metabolism of human platelets in collaboration with J. Muenzer were directed primarily on the role of non-heme iron in energy metabolism. It was found that the basal respiration of human platelets was greatly stimulated by iron-chelators and by bathophenanthroline, the specific reagent for iron analysis. Cyanide and antimycin completely inhibited the respiratory stimulation by these reagents. The effects are striking similar to release of respiratory control by uncouplers of oxidative phosphorylation in isolated mitochondria. These findings indicate that non-heme iron components participate in respiratory chain phosphorylations in human platelets, presumably at the mitochondrial locus.

Significance to Biomedical Research and to the Program of the Institute:

This investigation, because of its fundamental nature in attempting to elucidate vital biochemical mechanisms common to all aerobic cells, has obvious importance for biochemical research in general. This is shown by the numerous collaborative projects in which we have participated within and without the NIH during the past years. This investigation also provides a basis for understanding bioenergetic problems associated with parasitism. Studies of energy metabolism in parasites, in addition to their intrinsic and comparative interest, contribute toward a more rational understanding of the host-parasite relationship, and may provide information for chemotherapeutic exploitation.

Proposed Course of the Project: The parasite studies will be continued with emphasis on the mechanisms of electron transport and energy conservation in cestodes. Dual wavelength spectrophotometry will be used to identify and characterize the respiratory pigments of tapeworm mitochondria. Biochemical studies of E. histolytica will be focused on membrane biochemistry, particularly in regard to L-serine metabolism. If sufficient material is available, isolation of non-heme iron electron carriers will be attempted. Our collaborative investigation of the respiratory chain of Staphylococcus aureus will be continued.

The mammalian studies will be continued with emphasis on the mechanism of action of uncoupling reagents. Our studies with human skeletal muscle mitochondria will be resumed, and studies of animal cardiac muscle cells will be undertaken with Dr. J. Dvorak and Prof. L. Ernster (who will be a Fogarty Scholar-in Residence). Investigation of the energy metabolism in human platelets, as well as the work with cultured human fibroblasts from patients with acute, intermittent porphria has been terminated.

Honors and Awards:

E. C. Weinbach was invited to act as Chairman of a session on Energy Coupling at the Ninth International Congress of Biochemistry, July 1-7, Stockholm, Sweden.

E. C. Weinbach was an invited participant to the Symposium on Energy-Transducing Membrane Functions, July 8-9, Stockholm, Sweden.

Publications:

Weinbach, E. C. and Diamond, L. S.: *Entamoeba histolytica*: I. Aerobic Metabolism. *Exp. Parasit.* 35: 232-243, 1974.

Kark, R. A. P., Weinbach, E. C., Blass, J. P. and Engel, W. K.: Oxidative metabolism in small samples of normal and diseased human muscle. Clinical Studies in Myology. Part 2 of the *Proceedings of the Second International Congress on Muscle Diseases*, Perth, Australia, Excerpt Medica, (B. Kakulas, editor) 98-107, 1971.

Serial No. NIAID 123-E
1. Parasitic Diseases
2. Physiology and Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1974

Project Title: Biophysical Parasitology

Previous Serial Number: Same

Principal Investigator: James A. Dvorak

Other Investigators: None

Cooperating Units: Biomedical Engineering and Instrumentation Branch, DRS and Television Engineering Section, CC

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	19/12			19/12
Professional	12/12			12/12
Other	7/12			7/12

Project Description:

Objectives: To develop and utilize biophysical techniques for the study of parasitological problems of medical importance. Although major emphasis is still being directed toward a better understanding of the host-parasite interaction resulting in Chagas' disease, the techniques developed with this program are also being utilized in a study of the penetration kinetics of erythrocytes by malaria merozoites. The approach is two-fold: (1) to obtain a clearer understanding of the interaction at the cellular and subcellular level of the parasite with the host cell and (2) to relate this information to what is observed in a population of infected cells *in vitro* and, hopefully, the general course of events *in vivo*.

Methods Employed:

(1) *In vitro* cultivation

The controlled-environment culture system developed with this

program is used routinely to maintain in vitro steady-state physiologic conditions. This has permitted the recording of previously unobservable aspects of host-parasite interactions as well as the quantification, of the influence of diverse physical and/or chemical parameters on the interaction.

(2) Electro-optical microscopy

Several electro-optical microscopy systems developed with this program are used routinely to study host-parasite interactions at the cellular and subcellular levels. This includes high-resolution low-light-level video systems for qualitative studies as well as video intergrators and a digital interferometer for quantitative studies.

Major Findings:

(1) The interaction of Trypanosoma cruzi with vertebrate cells in vitro.

(a) Of the several possible approaches to the control or eradication of T. cruzi infections, chemotherapy has received the most attention. 3-Methyl-4-(5'-nitrofurfurylidene-amino)-tetrahydro-4H-1,4-thiazine-1,1-dioxide [Lampit], has been reported to be very active in the treatment of acute Chagas' disease and is in extensive clinical use in some areas. However, no serious attempt has been made to determine either the general mode of action of this compound or the specific stage in the parasite's life cycle which may be affected. Previous work of this program demonstrated the feasibility of studying the effects of metabolic inhibitors on the life cycle of T. cruzi in vitro. The same basic approach is being used to study the effects of Lampit on the life cycle of T. cruzi.

The effects of Lampit on the ability of trypomastigotes to penetrate vertebrate cells have been quantified. The cytotoxic drug level for bovine embryo skeletal muscle host cells (BESM) is 10^{-3} M. From 10^{-4} - 10^{-5} M, Lampit significantly inhibits (by 60-80%) but does not stop trypomastigotes from penetrating BESM cells. At levels of 10^{-6} - 10^{-9} M, Lampit has no significant effect on the penetration of BESM cells by trypomastigotes during the 4-hour time course of the experiments. Qualitatively, high concentrations of Lampit (10^{-4} - 10^{-5} M) decrease the motility of trypomastigotes. It is not yet known whether the effects of high Lampit levels on trypomastigotes is reversible. However, the most likely causes for the decreased motility are (1) inhibition of respiratory function of the parasite or (2) simply a disorganization of its subpellicular microtubules as would occur in the presence of Colchicine or vinblastin, etc.

The reproduction of intracellular amastigotes is inhibited at high Lampit concentrations but, contrary to previous reports, the parasites are not destroyed. At a sustained level of 10^{-5} M Lampit, the doubling time of the Ernestina strain of T. cruzi is increased to about 56 hours which is 400% greater than its normal doubling time of 13.8 hours at 35°C. The length of the lag period prior to reproduction does not appear to be affected, however.

It should be noted that the highest drug level which could safely be used in this study is about one log below levels reported as being trypanosidal by other workers. This has particular physiologic significance in view of the fact that high serum protein levels (10%) are present in the system. Furthermore, based on clinical data the 10^{-4} M level of Lampit corresponds, theoretically, to the upper limit which could be attained in man using recommended dosages. This level can not be sustained and often results in toxic side effects.

(b) Recent advances in support equipment for the controlled-environment culture system have permitted improved gas tension control. Changes in gas tension can now be made rapidly and reproducibly. Preliminary data indicate that there is a differential sensitivity to changes in gas tension between vertebrate cells and T. cruzi parasites. For example, when infected vertebrate cells are subjected to lowered O_2 tension, pronounced vacuolation of the kinetoplast-mitochondrion complex occurs. This effect can be reversed by restoration of normal O_2 tensions. Host cells are apparently not affected by equivalent changes in O_2 tension. Whether this change in the amastigotes is due to decreased O_2 tension directly or a result of the accumulation of metabolites (eg. lactate) is not known.

(c) Ultrastructural analysis of selected stages of the interaction between T. cruzi and vertebrate cells is in progress. Primary emphasis is directed toward an underatanding of events occurring during host cell penetration by trypomastigotes and the reorganization of trypomastigotes to amastigotes. It has not yet been definitely established that T. cruzi actively penetrates host cells. However, host cell membrane can not be found around trypomastigotes that have recently penetrated cells or at any subsequent stage. Thus, it would appear that if penetration is a passive process on the part of the parasite (i.e., endocytosis by the host cell) the membrane of the endocytotic vesicle is rapidly lost or destroyed. It is more likely that the parasites are never host cell membrane vesicle bound.

The transition from trypomastigote to amastigote involves several processes all apparently occurring simultaneously. Most marked is a geometric and morphologic transformation of the kinetoplast which correlates well with quantitative data on changes in the anisotropy of this structure. Furthermore, qualitative changes in electron density support previous data that there is no significant change in absolute mass of the parasite during reorganization but the mass per unit volume decreases.

(2) Malaria studies

It had been previously established that in vitro studies of the erythrocytic stage of malaria are feasible. However, the elucidation and recording of certain aspects of this interaction required the development of new electro-optical systems. Design details are now formalized to the point where a reliable low-light-level video system capable of maintaining

high spacial frequencies (high optical resolution) can be used routinely for the study of malaria merozoite-erythrocyte interactions. It has been established that:

1. The rupture of a schizont-infected erythrocyte is preceded by the coalescence of pigment in the residual body, swelling of the erythrocyte (probably due to an ingress of water and sodium), and a marked distortion of the erythrocyte caused by movement of the merozoites just prior to rupture of the cell.

2. Free merozoites are capable of intrinsic motion.

3. Merozoite penetration of erythrocytes is dependent upon the orientation of the merozoite with respect to the erythrocyte. Penetration only occurs if the end of the merozoite containing the paired organelles contacts the erythrocyte.

4. The initial contact between merozoite and erythrocyte results in marked deformation of the erythrocyte. The actual process of penetration is slow, requiring about 10 to 20 seconds.

5. Penetration of an erythrocyte by a merozoite results in swelling of the erythrocyte and can lead to lysis of the erythrocyte.

These last two points have been used as a basis to generate a physical-chemical model to describe the interaction between merozoites and erythrocytes.

Significant to Biomedical Research and the Program of the Institute:

(1) The study of host-parasite interactions at the cellular and subcellular level is a topic of critical importance. The outcome of this interaction directly influences the physiologic state and survival potential of both the host and the parasite.

A clearer understanding of the entire interaction has significantly improved the probability of finding biological "pressure points" for attempted interdiction of the parasite's cycle. For example, interdiction of the penetration phase of the interaction will totally interupt the natural life cycle of the parasite.

The general applicability of this research approach to the study of other human infectious diseases has been demonstrated by our ability to utilize our techniques in the analysis of the interaction of several protozoan parasites with vertebrate cells. The wealth of comparative information which is being accummulated should be of considerable value in the understanding of parasitic diseases in general.

(2) The methodology and research philosophy being developed are directly applicable to other areas of biomedical research. For example, the controlled-environment culture system developed with this program is

now in routine use in laboratories with such diverse interests as oncology, immunology, neurophysiology and morphogenesis. The video systems developed with this program are being used for analysis of macroscopic as well as microscopic images in basic research and diagnostic clinical-medical fields.

Proposed Course of the Project: Research will continue in an attempt to further our understanding of the interaction of T. cruzi with vertebrate cells in vitro and relate this information to the course of Chagas' disease in nature. Basic information obtained with the controlled-environment culture system and video systems will be utilized in more complex in vitro model systems.

The basic methodology and research philosophy developed with this program will be used for the analysis of other host-parasite interactions. Most specifically, studies of the interaction between malaria merozoites and erythrocytes will be continued and preliminary work on an in vitro model for the exoerythrocytic stage of the cycle will be started.

Honors and Awards:

An invited lecture entitled: "In vitro studies of the interaction between Trypanosoma cruzi and vertebrate cells" was presented by J. A. Dvorak at the University of Tennessee, Knoxville on May 24, 1974.

Invention: A controlled-environment culture chamber for high resolution light microscopy. Patent issued 10 April 1973. U. S. Patent No. 3,726,597.

Publications:

Miller, L. H., Dvorak, J. A., Shiroishi, T., and Durocher, J.: Influence of erythrocyte membrane components on malaria merozoite invasion. J. Exp. Med. 138: 1597 1601, 1973.

Dvorak, J. A. and Hyde, T. P.: Trypanosoma cruzi: Interaction with vertebrate cells in vitro. I. Individual interactions at the cellular and subcellular levles. Exp. Parasit. 34: 268-283, 1973.

Hyde, T. P. and Dvorak, J. A.: Trypanosoma cruzi: Interaction with vertebrate cells in vitro. II. Quantitative analysis of the penetration phase. Exp. Parasit. 34: 284-294, 1973.

Luban, N. A. and Dvorak, J. A.: Trypanosoma cruzi: Interaction with vertebrate cells in vitro. III. Selection for biological characteristics following intracellular passage. Exp. Parasit. (in press).

Dvorak, J. A. and Poore, C. M.: Trypanosoma cruzi: Interaction with vertebrate cells in vitro. IV. Environmental temperature effects. Exp. Parasit. (in press).

Dvorak, J. A., Schuette, W. H. and Whitehouse, W. C.: A simple video method for the quantification of microscopic objects. J. Microscopy (in press).

Serial No. NIAID 123-F
1. Parasitic Diseases
2. Physiology & Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Terminal Electron Transport in Parasites

Previous Serial Number: None

Principal Investigator: Dan R. Harlow

Other Investigators: Eugene C. Weinbach, Louis S. Diamond, Guillermo Pacheco, R. Frederic Knauft, H. G. Sheffield and F. A. Neva

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	12/12			12/12
Professional	12/12			12/12
Other				

Project Description:

Objectives: To elucidate the fundamental biochemical mechanisms of terminal electron transport by which parasites oxidize reduced compounds under both aerobic and anaerobic conditions.

Methods Employed:

(1) Cestode mitochondria. Mitochondria are isolated from adult Humenolepis diminuta. The life cycle is maintained in the laboratory using Tenebrio molitor and Tribolium spp. as intermediate hosts and Sprague-Dawley rats as definitive hosts. Adult tapeworms are harvested at 17 days post-infection by flushing from the intestine and washing them clean of intestinal contents with buffered Krebs-Ringer solution. The harvested tapeworms are homogenized and the mitochondria isolated by differential and sucrose density gradient centrifugation. The mitochondria obtained are much

"cleaner" than heretofore possible starting with a whole helminth homogenate. The isolated mitochondria are examined by low-temperature spectroscopy for presence of cytochromes and cytochrome-like compounds.

(2) Amoeba transhydrogenase. Axenically-grown Entamoeba histolytica are washed in buffered saline or buffered sucrose (L. S. Diamond) and examined for enzymatic activity either as intact organisms or sonicated organisms.

(3) Filariid oxidative metabolism. Microfilariae of Dirofilaria immitis harvested from the blood of infected dogs (Pacheco and Knauft) are incubated in the polarograph (Clark oxygen electrode) and the oxygen removal is measured. Substrate and specific inhibitor effects on oxygen removal are noted.

Major Findings:

(1) Cestode mitochondria: The life cycle supply system is now developed to the point of reliably producing adult H. diminuta tapeworms in quantities sufficient for the mitochondrial studies. H. diminuta mitochondria were isolated, lyophilized and sent to Dr. E. C. Weinbach while he was a guest worker in Dr. Lars Ernster's Laboratory at the University of Stockholm in 1973. The mitochondria were assayed for cytochromes there using the Aminco DW-2 dual wavelength spectrophotometer. Very definite absorbtions were seen in the cytochrome b region confirming the principal investigator's previous studies utilizing less precise instrumentation. Cytochrome a and cytochrome c appear to be present in smaller amounts. The briefness of Dr. Weinbach's visit prevented complete analysis of the tapeworm cytochromes but this study will soon be pursued in this laboratory utilizing an Aminco DW-2 spectrophotometer being purchased this year.

(2) Amoebal transhydrogenase: E. histolytica, while eukaryotic, lacks mitochondria and is either anaerobic or facultatively anaerobic. Transhydrogenase in other animal cells is located on the inner mitochondrial membrane and thus is intimitely associated with aerobic metabolism. Recent studies with the mitochondrial transhydrogenase indicate that it is involved with energy conservation. The transhydrogenase of the amoeba responds to palmitoyl-CoA and Ca^{2+} in a way similar to that of the mitochondrial enzyme and is thus likely also to be involved with energy conservation. The lack of a typical aerobic metabolism and the presence of transhydrogenase in the amoeba presents the following enigmatic situation: the mammalian mitochondrial transhydrogenase is involved in energy conservation and is always associated with a typical aerobic metabolism; the amoebal enzyme also is likely to be involved in energy conservation but is associated with an anaerobic or facultative anaerobic metabolism. Thus the amoeba may present a novel form of energy conservation differing from that of its mammalian host.

In collaboration with Dr. H. G. Sheffield, electron microscopical studies of differential centrifugation fractions of homogenized amoebae have shown enrichment of a membranous structure in the particulate fraction containing the highest specific activity of the transhydrogenase (110,000 X g).

Future structural studies are designed to determine definitively the structural locus of the enzyme.

(3) Filarial oxidative metabolism: Progress in this study haa been slowed by Dr. Pacheco's tragic illness and Dr. Knauft's departure from the laboratory. Prior to Dr. Knauft's departure, the "groundwork" of Dirofilaria immitis microfilarial oxidative metabolism was completed. The D. immitis microfilaria appears to be a typical aerobe; that is, glucose greatly stimulates oxygen utilization and this oxygen uptake is abolished totally by KCN as well as other standard inhibitors of aerobic electron transport. This finding emphasizes the wide variety of metabolic patterns among parasites, ranging from anaerobic to aerobic with many gradations between the two extremes reflecting the equally wide variety of ecological niches occupied by parasites.

Studies of human Wuchereria bancrofti microfilariae were initiated with parasites supplied by Dr. Neva and Al Gam from Tongan patients in the Clinical Center. Although the numbers of microfilariae available are small compared to the numbers of D. immitis microfilariae obtained, oxygen utilization studies have been initiated and cytochrome identifications should be possible using the new dual wavelength spectrophotometer soon to arrive in the laboratory.

Significance to Biomedical Research and the Program of the Institute:

The body of evidence accumulated on parasite biochemistry indicates primarily anaerobic metabolism. This is confused by the general observation that these parasites will utilize oxygen when present. The presence or absence of mammalian-type cytochromes is not yet satisfactorily established. Currently, the terminal electron transport mechanisms of parasites are not known - thus, whether they are similar to or different from the mammalian host's systems also is not known. It is of primary importance to determine these mechanisms both from the aspect of possible chemotherapeutic value and the aspect of intrinsic scientific merit.

Proposed Course of the Project: The study of the cytochromes of H. diminuta can proceed at a faster and more dependable rate with the arrival of the Aminco spectrophotometer. After definitive determinations of the tapeworm cytochromes, collaborative studies will be initiated with other staff members to determine cytochromes of the various other parasites now available in the laboratory.

It is expected the D. immitis microfilarial studies will be extended by Dr. Knauft's contributing some "volunteer" evening time for collecting microfilariae starting in mid-summer. The recently initiated W. bancrofti studies will be dependent upon the numbers of microfilariae available.

The amoebal transhydrogenase study has been the especially rewarding during the past year since it coincided with a heightened interest in mitochondrial transhydrogenase caused by recent studies into its energy

conservation aspects. It is hoped that the amoeba study can contribute to the understanding of the transhydrogenase reaction. The first published report on the amoebal work is now ready for submission to the journal. Future studies on the amoebal transhydrogenase will include aspects of energy metabolism in the amoeba.

Serial No. NIAID 125-A
1. Parasitic Diseases
2. Cell Biology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on Toxoplasmosis

Previous Serial Number: Same

Principle Investigator: Harley G. Sheffield

Other Investigators: Marjorie L. Melton

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	18/12			18/12
Professional	18/12			18/12
Other				

Project Description:

Objectives: The study of Toxoplasma gondii infections in cats to compare with the course of the disease in man; to determine the stages and sites of development of the parasite in the cat; to study development of immunity in cats and its relationship to the epidemiology of toxoplasmosis in man; to determine the nature of oocyst infections in man and animals.

Methods Employed: Cats are infected by feeding them cysts in mouse brain or oocysts isolated from feces of infected cats by sucrose flotation. Infectivity of concentrated oocysts in determined by feeding them to mice and subsequently checking for organisms or anti-toxoplasma antibodies. Tissues from infected animals are used for histological or ultrastructural studies. Cultured cells are used for in vitro studies of development of sporozoites which have been artificially excysted from oocysts and inoculated into cultures. In vitro studies are also used to study the effects of selected drugs on the parasites.

Major Findings: Studies on immunity to T. gondii in cats in its relationship to oocyst-producing reinfection have been continued. Partial to complete immunity to an oocyst-producing develops after initial infection and persists for an indefinite period. Of 40 cats studied, 12 produced oocysts upon reinfection. Eleven of these 12 cats were initially infected with strains of T. gondii which normally do not produce oocysts. These results suggest that oocyst production in the initial infection stimulates resistance to challenge at the intestinal level.

Two antibiotics, monensin and lasolacid, and a combination of ormetaprim and sulfamethoxime have been tested in vitro against T. gondii for comparison with earlier studies using pyrimethamine and sulfadiazine. The combination had effects similar to those seen with pyrimethamine and sulfadiazine. Monensin and, to a lesser extend, lasolacid inhibited penetration of cultured cells by T. gondii. Both compounds inhibited division of the parasites when added after penetration had occurred.

Additional studies on WC-1170, a Sarcocystis-like organism have been carried out in vitro. No recognizable differences have been noted between WC-1170 and T. gondii regarding their development in cultured cells. Infected cultures were examined by electron microscopy and one structural difference was seen (See 125-I). Since schizogony has only been found in epithelial cells of cat intestine attempts have been made to reproduce the in vivo findings using primary cat intestinal cells in vitro. No recognizable schizonts have been identified nor have any sexual stages been seen in cultures infected with either T. gondii or WC-1170.

Production of sexual stages of various coccidia in vitro has been unsuccessful except in cases where merozoites were removed from the host and inoculated into cell cultures. In an attempt to produce sexual stages of T. gondii cats were infected and portions of the intestine removed then incubated in culture medium. Merozoites freed from intestinal cells were collected from the medium and inoculated into cultures of several cell types. The negative results of this preliminary experiment were probably due to a very low yield of merozoites. Further studies are in progress.

Significance to Biomedical Research and the Program of the Institute:

The identification of the cat as the natural host for T. gondii provides a new insight into the epidemiology of toxoplasmosis and could explain the wide-spread existence of anti-toxoplasma antibodies in man and animals. In addition, the linking of T. gondii, whose classification has been uncertain for many years, to the coccidia provides the investigator with many model systems which could be used for comparison purposes in the study of toxoplasmosis in normal and abnormal hosts. The cat's role as a reservoir animal may be significant in the cosmopolitan distribution of the disease. Information on the immunological aspects of the disease in cats would show the possibility of reinfection and may provide new leads relative to the cat being an important reservoir of the organism. Further elucidation

of the various stages of the life cycle in the cat would contribute to a better understanding of the disease in man. Basic information on the biology of the organism and relationship to other protozoa could give some explanation for the parasite's unusual lack of host specificity and unique means of multiplication.

Proposed Course of Project: Cultivation of T. gondii in vitro will be continued. Miltiplication of the organism under various normal and abnormal conditions will be studied in attempts to determine why the parasite divides by schizogony in cat intestine but by endodyogeny in other cell types. The immunological aspects of the disease in the cat will be examined to supplement existing data on the importance of cats as reservoir hosts. Studies on related organisms will be continued for comparative purposed and to examine the possibility that other coccidia would be infective for man.

Honors and Awards:

Editor, Proceedings of the Helminthological Cociety of Washington

Consultant, Journal of Parasitology

Member, Meeting Site Selection Committee, American Society of Parasitologists.

Representative to National Research Council, National Academy of Sciences for Society of Protozoology.

Cochairman, Section on Life Cycles of Coccidia, Toxoplasmidae and Sarcocystidae, Third International Congress of Parasitology, Munich, 1974.

Serial No. NIAID 125-B
1. Parasitic Diseases
2. Cell Biology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: In-vitro cultivation of Coccidia

Previous Serial Number: Same

Principal Investigator: Franklin A. Neva

Other Investigators: Milford Lunde

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	2/12			2/12
Professional	2/12			2/12
Other				

Project Description:

Objectives: Since Toxoplasma gondii has recently been found to be a coccidian type parasite it seems appropriate to consider the potential of other coccidia as human pathogens.

Assessment of the pathogenicity of other coccidia for man is particularly needed since practical serologic tests to indicate infection with these organisms are virtually non-existant. Therefore, if various coccidia could be easily cultivated in-vitro such a system might provide antigens for serologic tests or a system for assay of antibody against the organism. In addition, basic information about the biologic behavior of these organsims might be gained by their study under conditions of in-vitro cultivation in cell cultures.

Methods Employed: Standard methods of cell culture with primary as well as cell-line cultures are used. Inoculated cultures are observed directly for evidences of parasite growth and cultures or material from

harvested fluids are fixed and stained. Oocysts of Isospora canis were kindly provided by Dr. Kenneth Todd of the University of Illinois. Dr. Gordon Wallace of Pacific Research Section provided oocysts of the murine Sarcocystis strain WC1170. Oocysts are permitted to sporulate and excysted with the same techniques as has been used for Toxoplasma. Animals are inoculated and tissues are obtained for histologic study by standard techniques.

Major Findings: 1. All work was restricted to the Sarcocystis-like organism, strain WC-1170, discovered by Dr. Gordon Wallace of the Pacific Research Station. Dr. Gerald Aronheim, a guest worker was able to infect cats by feeding them infected mouse tissues, and to recover the toxoplasma-like oocysts from infected cat feces. Repeated attempts to grow the WC-1170 organism in cell cultures gave only abortive infections, which died out within 4-6 weeks. Mice inoculated with oocysts or excysted sporozoites show only scanty and irregular presence of cystic organisms in the muscles and in only about half of the inovulated mice can low levels of Toxoplasma dye-test antibody be demonstrated.

2. In attempts to propagate the WC-1170 organism in cell cultures, some of the passage-line cells exhibited a change in morphology and appear to have undergone transformation. The transformation phenomenon, for WI-38 cells, shows preliminary evidence of being transmissible by cell-free materials, but alternative explanations for this cannot yet be excluded.

Significance to Biomedical Research and the Program of the Institute.

The possibilities of being able to prepare convenient antigens with the coccidial agents tested so far in infected cell cultures does not appear promising. Dr. Aronheim, the guest worker on this project, hopes to pursue some aspects of the work on return to Duke University--he wanted specifically to look for evidence of an anti-coccidial interferon in cultures that fail to support growth of the WC-1170 organism in-vitro.

Proposed Course of the Project: We will confine our interest in trying to establish whether the "transformed cells" arising in the course of these experiments contain a cell-free transmissible factor.

Serial No. NIAID 125-C
1. Parasitic Diseases
2. Cell Biology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Isolation of Circulating Antigen in Experimental Mammalian Toxoplasma Infection

Previous Serial Number: None

Principal Investigator: Dr. Richard Raizman

Other Investigators: Dr. Franklin A. Neva

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	12/12			12/12
Professional	12/12			12/12
Other				

Project Description:

Objectives: Because of the limitation of antibody tests to document acute Toxoplasma infection, this study was undertaken to determine whether an antigen marker might be present in the sera of animals acutely infected with Toxoplasma gondii.

Methods Employed: Mice and rabbits infected with R_H strain Toxoplasma were bled at various times during the course of acute Toxoplasma infection. Sera from these animals were examined for the presence of antigen by 1) countercurrent immunoelectrophoresis (C.E.P.); 2) Agar-gel double diffusion (A.G.D.D.); and in the rabbit only; 3) affinity chromatography, using CNBr-activated sepharose. Blood was simultaneously subinoculated into mice for determination of parasitemia.

Major Findings: In mice, who uniformly die within four days of intraperitoneal inoculation with R_H Toxoplasma, circulating antigen could be identified by C.E.P. and A.G.D.D., on days 2, 3, and 4 of infection. Parasitemia was also present during days 2, 3, and 4 but no organisms were visible on microscopic examination of blood smears.

Characteristics of mouse-obtained circulating antigen include stability at 37°, but not at 56°; stability to freezing and lyophilization; molecular weight > 100,000 by Amicon filtration; and elution in the void volume of sephadex G-200 column.

In rabbits, who die within eight days of sub-cutaneous R_H Toxoplasma inoculation, circulating antigen is not detected, in most cases, by C.E.P. or A.G.D.D. Affinity chromatography, however, allows isolation of circulating antigen on days seven and eight of infection, during which time parasitemia is evident by mouse subinoculation, but not on microscopic examination of blood.

The rabbit and mouse circulating antigen form lines of identity with reference mouse Toxoplasma peritoneal exudate antigens, but do not show identity with normal mouse or normal rabbit sera.

Significance to Bio-medical Research and the Program of the Institute:

That a serologic marker does exist in acute experimental Toxoplasma infection has the possibility of eventually providing an antigen test for Toxoplasma in humans. Since the current antibody tests confirm only previous exposure to Toxoplasma, but not necessarily activity of the disease, an antigen test would more accurately define the acute stage of infection in humans.

Proposed Course of the Project: With purified antigen obtained from affinity chromatography of infected rabbit sera, a more specific hyperimmune antisera will be employed for further specificity. Eventually, a more sensitive radio-immune assay will be attempted for identification of antigen from presumed active human toxoplasmosis.

Serial No. NIAID 125-D
1. Parasitic Diseases
2. Cell Biology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Pathogenesis of Disease (Chagas' Disease) Resulting from Infection with Trypanosoma cruzi

Previous Serial Number: 132-0

Principal Investigator: Franklin A. Neva

Other Investigators: Dr. Gustavo Parajon of Hospital Baustista in Managua, Nicaragua

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	10/12			10/12
Professional	1/12			1/12
Other	9/12			9/12

Project Description:

Objectives: To elucidate some of the factors that determine why infection with T. cruzi is associated in some individuals with either acute or chronic disease. To develop improved serologic procedures for diagnosis of infection and/or disease due to T. cruzi.

Methods Employed: A number of strains of trypanosomes are maintained in culture, others by syringe passage, and some are maintained in serial passage alternating between mice and vector bugs. The biologic characteristics of strains kept in mouse-bug passage are also tested at intervals by checking peripheral parasitemia and histopathology of mice. Colonies of two species of triatomid bugs are maintained. One strain of T. cruzi is passaged in cell cultures of embryonic beef tissue, and tissue phases of the parasite (mainly amastigotes and trypomastigotes) are harvested for antigen. Complement-fixation and gel-diffusion tests for detection of antigen-antibody reactions are done in a standard manner. Antisera to

different T. cruzi antigen preparations are obtained by immunization of animals and by collection from patients. Follow-up examinations of patients surveyed earlier in the field have been conducted by Dr. Parajon for clinical evaluation and diagnosis of their eardiac status.

Major Findings:

1. Field study in Nicaragua. Dr. Neva went to Nicaragua in the fall of 1973 to meet with Dr. Parajon for a final clinical evaluation of the heart disease cases. All of the information is now at hand to write up our experience in community assessment of relative prevalence of Chagas' heart disease and the serologic survey for T. cruzi infection in Nicaragua.

2. T. cruzi antigen from infected cell cultures: Our work on the experience with the CF antigen from infected cell cultures is in preparation for publication. Studies with the cell culture antigen in gel diffusion tests have been temporarily halted because of the press of other work but will be resumed shortly.

3. Comparative behavior of different strains of T. cruzi: Preparation of manuscripts of this work still has not been completed.

Significance to Biomedical Research and Program of the Institute:

Our experience in community assessment of prevalence of Chagas' heart disease in Nicaragua has been described in verbal reports to others and is being utilized by the Harvard-Wellcome group in Brazil. The Pan American Health Organization has expressed interest in supporting similar studies in other areas of Latin America. The information on comparative behavior of different strains of T. cruzi and biologic differences between infective stages from bugs vs. cultures continues to be utilized and made available to other investigators in LPD even though it has not been published. The comparative usefulness of the cell culture antigens of T. cruzi in serodiagnosis are still being evaluated.

Proposed Course of the Project: Much of the work simply awaits preparation into manuscript form and submission for publication. Gel-diffusion studies with the cell culture antigen will be resumed when time permits. The possibility of carrying out a serologic survey for T. cruzi infection with sera already collected by others in Texas is being explored.

Neva, F. A. American Trypanosomiasis in Diseases Transmitted from Animals to Man. 6th Ed. Edited by Hubbert, W. T., McCulloch, W. F., and Schnurrenberger, P. R. Publisher, Charles C. Thomas, 1974.

Serial No. NIAID 125-I
1. Parasitic Diseases
2. Cell Biology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Electron Microscope Studies on Parasites

Previous Serial Number: Same

Principal Investigator: Harley G. Sheffield

Other Investigators: None

Cooperating Units: Animal Parasitology Institute, ARS-USDA, Beltsville, Maryland

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	18/12			18/12
Professional	6/12			6/12
Other	12/12			12/12

Project Description:

Objectives: To determine the fine structure of various parasites in relation to their taxonomy, life cycles, reproduction and other physiological functions.

Methods Employed: Parasites are obtained from their host animals or culture at the developmental stage desired or after appropriate experimental procedures. The parasite tissue is processed, sectioned and then studied with the electron microscope and/or cytochemical methods.

Major Findings: Sporozoite structure. Studies using various species of coccidia are being conducted to determine to comparative ultrastructure of their respective sporozoites. Sporozoites of Toxoplasma gondii, WC-1170 a Sarcocystis-like organism (see 125-A), Isospora canis, and Sarcocystis fusiformis have been examined. Characteristic organelles such as the conoid, rhoptries, micronemes, a two-membrane pellicle with underlying microtubules, micropore and typical protozoan mitochondria were identified. In addition,

globular structures believed to be storage material were present in all sporozoites except those of T. gondii. This finding could be significant since T. gondii, which has a different type storage granule, is apparently more pathogenic and has a wider host range. In conjunction with the sporozoite study, oocyst and sporocyst walls are being examined to determine their structure, origin and mechanism of rupture.

In vitro study of WC-1170 organisms has been expanded to include ultrastructural examination of developing organisms for comparison with T. gondii. Although penetration and division rates have not been determined the growth pattern and ultrastructure of WC-1170 infecting monkey kidney cells are consistent with those of T. gondii. One exception is the presence of storage material (see above) in the WC-1170 organisms. The material is abundant in the invading parasites but disappears within 24 to 48 hours after cell invasion.

Studies in collaboration with Dr. Ronald Fayer, Animal Parasitology Institute, ARS-USDA, Beltsville, Maryland, are being continued. Oocyst stages of Sarcocystis fusiformis have been identified in the intestinal tract of experimentally infected dogs. Younger stages, perhaps gametocytes, have also been found and are being studied. Further work is necessary to determine the developmental cycle of the sexual stages. Such information is important in understanding the role of the dog in transmission of Sarcocystis to cattle and potentially to man.

Cysts of Giardia lamblia, a human intestinal flagellate, have been collected and concentrated for electron microscope studies by Dr. Bjorvatn (see). The cysts are enclosed by a thick cyst wall and contain a somewhat undifferentiated cytoplasm. Prominent features include 4 nuclei, 8 basal bodies and their associated flagella and a peripheral zone of vesicles. Scattered in whorls are numerous rod-like structures which are believed to be precursors of the sucking disk found in the trophozoite form. Studies are being continued to determine the cytoplasmic changes occurring during the trophozoite-cyst portion of the life cycle.

Assistance has been provided for ultrastructural examination of parasites and pathological tissues of snails (see 121-C). Assay of various fractions of biochemical preparations of Entamoeba histolytica has also been carried out (see 123-C and 123-F).

Significance to Biomedical Research and the Program of the Institute:

Continued investigation of the similarities between Toxoplasma gondii and other coccidia provides new leads to the study of the biology and epidemiology of the former. Study of the differences of development in the natural host compared to that in man and other animals could provide insight into the mechanism of host specificity in this parasite. Study of related organisms will provide additional information which could allow a better understanding of the structure of T. gondii and its relation to the functioning of the organism.

Proposed Course of the Project: Studies on the endogenous stages of T. gondii in the cat intestine under normal and experimental conditions will be continued. Development will also be studied in vitro as techniques permit. Mechanisms of penetration will be studied and supplemented with information obtained using other parasites. Other toxoplasma-like organisms will be studied as material is available and compared with existing data. Studies of the cyst of Giardia lamblia and its formation will be continued to provide information of the role of the organism as a human pathogen. A portion of the time will be used to provide other investigators with electron microscopic assistance when their needs arise.

Serial No. NIAID 125-K
1. Parasitic Diseases
2. Parasite Growth and Differentiation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Amebiasis studies in India

Previous Serial Number: Same

Principal Investigator: Louis S. Diamond

Other Investigators: Milford N. Lunde

Cooperating Units: Dr. L. N. Mohapatra, All-India Institute of Medical Sciences, New Delhi, India (PL 480 Project Agreement No. 01066-1).

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	2/12			2/12
Professional	2/12			2/12
Other				

Project Description:

Objectives: To evaluate an intradermal test for screening of amebic infections using an antigen produced from axenically cultivated Entamoeba histolytica; to isolate strains of Entamoeba from the indigenous population by means of in vitro techniques and to screen them for presence of E. histolytica-like Laredo type amebae; to study the pathophysiology of clinical manifestations of chronic intestinal amebiasis and amebic hepatitis; to investigate the possible relationship between amebic infection and non-specific ulcerative colitis.

Methods Employed: Clinical and parasitological evaluations using classical techniques. Serological tests using intradermal inoculation, indirect hemagglutination, agar-gel, counter current immunoelectrophoresis. Stool, liver and pleural exudate culture. Histopathologic study using light,

phase and electron microscopy.

Major Findings: The skin test antigen Histolyticin has been titrated in two groups of human subjects: a) Healthy individuals-controls, b) individuals with proven clinical and parasitological diagnosis of invasive amebiasis (i.e., liver abscess or acute dysentery). Doses of 0.25, 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0 ug amebic protein were assayed, and in as far as possible administered to the same individual. Briefly, the results were as follows: In healthy controls, doses of 0.25 and 0.5 ug of Histolyticin gave uniformly negative results. With increasing doses of 1.0 to 6.0 ug false positives increased from 3.6 to 10.4%. In invasive amebiasis, no positives were identified at dose levels of 0.25 and 0.5 ug, and no more than 38% positives were detected with the 2.0 ug dose. In contrast doses of 4.0 to 8.0 ug identified more than 68% of the positives, with the 6.0 ug dose picking up more positives than the 4.0 ug dose. There was no difference between the number of positives picked up by the 6.0 and 8.0 ug dose.

In general there was a rough correlation of 80% between the skin test and IHA results.

Tonicity and temperature studies performed on 20 culture isolates failed to uncover any E. histolytica-like Laredo type amebae in the samples.

Liver biopsies from 6 patients with non-supperative hepatic amebiasis (amebic hepatitis) (IHA-Pos.) and one healthy subject (IHA-Neg.) were studied by electron microscopy. Electron microscopy revealed the presence of diffuse hepatocellular injury whereas light microscopy did not show evidence of hepatitis in non-suppurative hepatic amebiasis. Mitochondria and the endoplasmic reticulum were the targets of organelle pathology. Paracrystalline, filamentous, and tubular inclusion bodies were observed in the matrices of large and bizarre shaped mitochondria in 5 of the 6 patients. The endoplasmic reticulum was dilated and hypertrophied in all. No amebae or virus associated with amebae were observed in the liver biopsy specimens.

In order to determine the relationship of amebic infection and non-specific ulcerative colitis three parameters were employed. 1). Three stool samples were examined using classical techniques, 2) the response to anti-amebic treatment and 3) the indirect haemagglutination test and the intradermal test.

Twenty cases who were diagnosed non-specific ulcerative colitis on established criteria were studied. In none of these cases could E. histolytica be demonstrated in spite of three consecutive stool examinations. In 19 individuals the stool did not reveal any intestinal parasites and in only one case cysts of E. hartmanni and Giardia lamblia and ova of Trichuris trichura were present. The anti-amebic treatment given to these patients failed to bring about amelioration of symptoms. The IHA test was performed on all twenty cases and the test was negative in all individuals except one who showed a titer of 1:18 which is not considered diagnostic in our laboratory. The ID test using 6.0 ug dose was done on eight individuals and was negative in all of them.

The results therefore do not suggest any association between non-specific ulcerative colitis and amebic infection of the colon.

Significance to Biomedical Research and the Program of the Institute:

The present titration studies taken together with previously reported studies on the clinical evaluation of Histolyticin in India and at the NIH indicate a dose of 6.0 ug amebic protein to be the most practical for detection of extra-intestinal amebic disease in a clinical setting.

The studies on non-specific ulcerative colitis, a common ailment in India, appear to rule out an origin in an amebic infection.

The electron microscope studies on liver biopsy material from cases of amebic hepatitis shed new light on this controversal entity.

Proposed Course of the Project: The Project closes June 30, 1974. The data gathered is being prepared for publication.

Serial No. NIAID 125-P
1. Parasitic Diseases
2. Cell Biology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1974

Project Title: Immunological studies on toxoplasmosis and other parasitic diseases

Previous Serial Number: Same

Principal Investigator: Milford N. Lunde

Other Investigators: Louis S. Diamond, Kendall G. Powers and William E. Collins

Cooperating Units: Primate Malaria Unit, Chamblee, Georgia

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	19/12			19/12
Professional	14/12			14/12
Other	5/12			5/12

Project Description:

Objectives: To evaluate and improve the indirect hemagglutination test (IHA) for malaria, to explore the feasibility of employing horseradish peroxidase in the sero diagnosis of parasitic diseases.

Methods Employed: Soluble malaria IHA antigen is prepared from Plasmodium knowlesi schizonts which have been separated from non-parasitized erythrocytes by passage through a Ficoll gradient. Sephadex G-200 columns are used to fractionate antigens which are used in the IHA test.

Horseradish peroxidase conjugated to goat anti human IgG globulin or goat anti human IgM globulin is used in a procedure similar to that used for the indirect fluorescent antibody test.

Sera were obtained from: a) Clinical Center, NIH, b) PL-480 project 01066-1, New Delhi, c) Primate Malaria Unit. Chamblee, Ga.

Major Findings: No differences in IHA reactivity using human sera were observed between P. knowlesi, P. falciparum, and P. vivax antigens. The appearance of detectable IHA antibody seems to be determined by the duration of the primary parasitemia. With 14 patients having a primary parasitemia of under 6 days only 4 or 14% had detectable IHA titers whereas 16 of 21 (76%) with parasitemia of more than 9 days had positive IHA titers >1:18.

Centrifugation at 10,000 x G of schizonts following a single freeze-thaw yields an antigen supernatant which is equal to or better than any obtained by additional procedures such as sonication, French press cell rupture or multiple freeze-thaw cycles. 75-90% of the available antigen is obtained by this single one step procedure.

Anti-human IgG globulin labeled with horseradish peroxidase has been successfully used to detect antibody to Toxoplasma, E. histolytica and Trypanosoma cruzi. 53 of 53 sera positive in the IHA test for toxoplasma at a titer of 1:54 or greater were positive at 1:16 or greater using horseradish peroxidase. 28 of 32 sera negative in the toxoplasma IHA test were negative with horseradish peroxidase. 24 of 32 sera with a clinical diagnosis of invasive amebiasis had positive indirect horseradish peroxidase titers (IHRP) of 1:16 or greater while 0 of 21 uninfected controls were negative. The results of the IHRP test with T. cruzi are encouraging although a series of sera from documented cases has not been tested.

See NIAID 132-C for studies on the effectiveness of chlorinated lincomycin analogues against the cyst stage of toxoplasma.

Significance to Biomedical Research and the Program of the Institute:

The search for improved sero-diagnostic tests in parasitic diseases and a better understanding of how these relate to the clinical situation and the experimental host in the laboratory will be of value not only to the clinician but also to the investigator seeking a better understanding of host parasite relationship and the immune factors involved.

The antigens which are currently used in the malaria IHA test are not standardized either as to the Plasmodium species used or in the method of preparation. A standardized antigen is essential to any serological procedure used in sero diagnosis or in field epidemiological studies. It is being suggested by some investigators that placental P. falciparum antigen be used in the IHA test. The difficulties in getting large standardized quantities of antigen from human placental tissue are obvious. It is hoped by demonstrating that a good malaria antigen, capable of standardization, can be obtained from primate Plasmodium it will not be necessary to use human placental tissue.

Proposed Course of the Project: Work to further the development and evaluation of horseradish peroxidase labeled antibodies and antigens for use in clinical and experimental parasitology will be continued.

An effort will be made to standardize the malaria IHA antigen and to determine whether the major part of reactive material originates in the erythrocyte cytoplasm outside the parasite or from the parasite itself.

It is planned that a cooperative project with Dr. Charles Alford, Dept. of Pediatrics, University of Alabama School of Medicine will be initiated to study the role of toxoplasma specific IgM antibody in pregnancy on fetal development.

A study will also be initiated toward the possible development of an antigen which can be used in the sero diagnosis of Pneumocystes carinii, especially in immunosuppressed patients.

Publications:

Lunde, M. N.: Laboratory methods in the diagnosis of toxoplasmosis. Health Lab. Sci. 10: 319-328, 1973.

Serial No. NIAID 132-A
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Mechanism of Invasion of Malarial Merozoites

Previous Serial Number: None

Principal Investigators: Louis H. Miller and James A. Dvorak

Other Investigators: Tsugiye Shiroishi and Bruce K. Schrier, NICHHD, Behavioral Biology Branch

Cooperating Units: Dr. John Durocher, Walter Reed Army Institute of Research, and Dr. Masamichi Aikawa, Institute of Pathology, Case Western Reserve University, Cleveland, Ohio.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	20/12			20/12
Professional	8/12			8/12
Other	12/12			12/12

Project Description:

Objectives: To understand the mechanism by which the malarial merozoite gains entry into the red cell and to evaluate methods for blocking invasion.

Methods Employed: Schizont infected red cells of Plasmodium knowlesi were separated from plasma, platelets and uninfected red cells. Uninfected red cells were then added to the schizonts, and the mixture was placed in a Dvorak chamber at 35°C. Culture media was perfused over the cells and rupture and reinvasion of red cells were observed by a Leitz inverted interference microscope. Quantification of invasion was performed in Giemsa stained thin smears of the cells from the chambers. Uninfected red cells placed in the chamber included red cells of different species and enzymatically altered red cells. The influence of red cell membrane components on invasion was studied.

The interaction of immune sera with merozoite was observed in a Dvorak-Stotler Chamber and the merozoite membrane was studied by transmission electron microscopy after exposure to immune and normal sera.

Major Findings: 1) Observation of the interaction between merozoites and red cells. Exocytotic vesicles form on the red cell membrane immediately before shcizont rupture. After rupture, merozoites must contact red cells within 10 minutes. The following sequence of events occurred during invasion:

1) Reversible attachment between the anterior end of the merozoite and the red cell.

2) Diffuse perturbation of the red cell.

3) Endocytosis of the merozoite.

2) The effect of enzyme alteration of red cell surfaces on P. knowlesi invasion. Chymotrypsin- and pronase-treated human red cells were refractory to invasion by merozoites; invasion was not inhibited by trypsin- or neuraminidase treatment. Invasion of rhesus erythrocytes was unaffected by pretreatment with these enzymes. The effect on membrane proteins on acrylamide gels was similar for rhesus and human red cell ghosts. Human glycoproteins were more susceptible to trypsin and chymotrypsin. However, invasion was uninfluenced by trypsin removal of all stainable glycoprotein in human red cells. Complete removal of acetylcholinesterose activity from rhesus red cells did not influence invasion.

3) The effect of human red cell components on invasion. Purified sialoglycoprotein and concentrated pronase and chymotrypsin digests did not block invasion by merozoites.

4) Merozoite surface characteristics. A fuzzy coat completely surrounds merozoites. The coat is removed by immune sera. Immune sera agglutinates merozoites by interaction with the fuzzy coat. This coat is pushed aside during invasion.

5) Effect of immune sera on red cell invasion. (See Annual Report NIAID 132-B).

Summary: 1) Invasion is initiated by attachment of merozoites to red cells. The component in the red cell responsible for attachment is not sialic acid or acetyl cholinesterose and does not appear to be sialoglycoprotein. Studies continue on the nature of the receptor.

2) The stimulus for diffuse perturbation and endocytosis is unknown.

3) The red cell mechanism of endocytosis is unknown.

4) Merozoites have a large fuzzy coat that is removed during invasion.

5) Immune sera does not neutralized receptor on merozoites but agglutinates merozoites by cross linking the fuzzy coat.

Significance to Biomedical Research and the Program of the Institute:

The identification of the receptor on erythrocyte membranes might permit isolation of the component on the merozoite that initiates invasion. It may then be possible to immunize the host against this site on the merozoite in order to block erythrocyte penetration.

Proposed Course of the Project: 1. Identify red cell receptor. 2. Identify substance that initiates endocytosis. 3. Determine structure and function of the fuzzy coat. 4. Attempt to grow P. vivax in culture in order to explore the question of why P. vivax does not infect most American blacks and West Africans.

Honors and Awards:

Member, Clinical Research Committee, National Institute of Allergy and Infectious Diseases

Member, Ad Hoc Committee to review intramural research and contract proposals in Tropical Diseases for the Army R & D Command at WRAIR.

Member, Steering committee, for organizing a work shop on vaccines for malaria for the National Research Council.

Participant, Symposium on antigenic variation in protozoa, WRAIR, November 30, 1973.

Invited speaker by the American Red Cross for symposium on infectious diseases transmitted by blood transfusion, May 8, 1974.

Publications:

Miller, L. H., Dvorak, J. A., Shiroishi, T., and Durocher, J. R.: Influence of erythrocyte membrane components on malaria merozoite invasion. J. Exp. Med. 138: 1597-1601, 1974.

Miller, L. H., Powers, K. G., Finerty, J., and Vanderberg, J. P.: Difference in surface charge between host cells and malarial parasites. J. Parasit. 59: 925-927, 1973.

Chien, S., Cooper, G. W., Kung-ming, J., Miller, L. H., Howe, C., Usami, S., and Lalezari, P.: A case of n-acetylneuraminic acid deficiency in erythrocyte membranes: Biophysical and Biochemical correlates. Blood (in press 1974.

Cooper, G. W. and Miller, L. H.: Propanoic acid-ferric oxide hydrosols: Differential cell surface binding and its relation to membrane lipid. J. Histochemistry and Cytochemistry. In Press, 1974.

Herman, R., and Shiroishi, T.: Plasmodium gallinaceum: Selective immunosuppression by cyclophosphamide in preerythrocytic malaria. Exp. Parasit. 34: 295-305, 1973.

Herman, R., Shiroishi, T., and Buckler, C. E.: Visual interference of exoerythrocytic forms of malaria (Plasmodium gallinaceum) in ovo. J. Inf. Dis. 128: 148-155, 1973.

Serial No. NIAID 132-B
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on Immunity and Pathology of Malaria and Other Parasitic Diseases.

Previous Serial Number: Same

Principal Investigators: Kendall G. Powers and Louis H. Miller

Other Investigators: M. Lunde and Philip R. B. McMaster

Cooperating Units: Laboratory of Microbial Immunity, NIAID

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	16/12			16/12
Professional	6/12	1/12		7/12
Other	9/12			9/12

Project Description:

Objectives: To study the immune response and pathological consequences of acute and chronic malaria and other parasitic diseases.

Methods Employed: Various host-parasite models are used in these studies including Plasmodium knowlesi and P. cynomolgi in rhesus monkeys, P. falciparum in owl monkeys and P. berghei and P. vinckei in rodents.

We are attempting to identify and characterize different malarial antigens and their respective antibodies in order to determine their relative role in the overall immune response. a) Antigenic variation in malaria is being studied in vivo and in vitro using the schizont agglutination test to monitor antigenic differences between parasite population during acute and chronic infections in individual nonhuman primate hosts. b) The effect of host immunity on the invasion of erythrocytes by merozoites is being investigated in vitro. Immune or normal monkey sera are added to culture media

containing mature schizonts and uninfected red blood cells. The number of cells invaded by the merozoites are counted. The effect of complement on this process also is being evaluated. For details of this system refer to Annual Report 132-A. c) The indirect hemagglutination test (IHA) for the detection of specific malarial antibodies is being evaluated and procedures are being devised to improve the antigen used in this test (For details refer to Annual Report 125-P).

The induction of immunologic paralysis was studied in 16 nonhuman primates using relatively small and large doses of pneumococcal polysaccharides. For theoretical reasons, as well as for setting practical immunization doses and schedules, it was of interest to determine whether such a phenomenon could occur in species other than the mouse. For this purpose rhesus and owl monkeys were injected with 10 to 10,000 α of Type III pneumococcal polysaccharide (SSS-III). In addition, some monkeys given 10 to 50 α were later challenged with 1,000 to 10,000 α. Sera were collected at suitable intervals and tested for the presence of antibody and, in selected instances, for antigen using radioimmunoassay procedures.

Major Findings: a) Several modifications have been made in the schizont agglutination test (SAT) as described by Brown. By using the microtiter technique we are able to use 0.025 ml amounts of serum and diluted antigen rather than the 0.5 ml amounts previously required. By use of ficoll gradients we are able to separate malaria-infected erythrocytes (antigen) from uninfected cells and the majority of the infected cells are recovered at an optimal stage of development for use in the SAT. A technique has been devised to preserve schizont-infected erythrocytes so that antigenic material can be standardized and stored over considerable periods of time. This assures a uniform antigen source for various studies and allows for the "banking" of stabilates that can be used in laboratory and field studies. Previously, schizonts had to be used within 24 hours after collection. We have confirmed Brown's findings that antigenically distinct populations develop in malaria infections and this may be the process by which the parasite survives the immune mechanism of the host. Sequential serum titers from monkeys infected with chronic P. knowlesi indicate that there may be two types of immunity. One is specific for each variant and the other transcends the varients and partially suppresses parasite multiplication. Although each recrudescence usually produces an antigenically different population, in some instances there is complete serologic similarity between populations that occur several months apart. b) Immune sera blocked invasion of red cells by agglutination of the merozoites. Immediately after rupture from the infected red cell, the receptor for invasion on the merozoite was not neutralized but interacts normally with the red cell. Addition of complement to immune sera does not further reduce the invasion rate. Complement does cause merozoite lysis but at a time when the merozoite could no longer invade red cells. Immunity, as measured by this serologic test (blockage of invasion) does not always correlate with functional immunity. c) It has been found that 75 to 90 percent of the soluble antigen in P. knowlesi-infected erythrocytes can be obtained using a single freeze-thaw procedure. This recovery compares favorably with techniques requiring more elaborate equipment such

as sonication, french press cell rupture etc. Antigens thus prepared were as good or better in detecting antibodies for P. falciparum or P. vivax than antigens produced by other methods. It was determined that there is no advantage in P. falciparum antigen, which is often difficult to obtain, over P. knowlesi antigen in the detection of falciparum antibodies in human sera.

Although the injection of foreign materials into animals usually stimulates antibody formation, certain exceptions have been observed. An appropriate small dose of Type III polysaccharide immunized monkeys and resulted in a rise in their antibody levels whereas a large quantity of the polysaccharide reduced their serum antibody. Monkeys given 10 to 50 α of Type III preumococcal polysaccharide responded by increasing the antibody level in their serum. By contrast, in those given 10,000 α the serum antibody was markedly reduced and generally remained at a low or undetectable level for weeks.

Significance to Biomedical Research and the Program of the Institute:

During the malarial infection, the host produces antibodies to infected red cells that are coated with malarial antigens. Probably of equal importance for functional immunity are the antigens present on the surface of merozoites. Circulating antibody, with or without complement, could effectively destroy the merozoite or block its infectivity to erythrocytes.

If there are antigenic variants in malaria then the problem of immunization becomes more difficult, even if the immunogens can be identified. It is hoped that these studies will develop methods to classify the antigenic varients, to separate and identify the antigens on the surface of the infected red cell and to identify the stage of the parasite responsible for malarial immunity.

Proposed Course of the Project: Work on the application of the schizont agglutination test in studying malarial immunity will be continued. Other investigations will involve the identification and characterization of the antigenic components that are on the erythrocyte surface, within the red cell between the membrane and the parasite and contained in the free parasite.

Honors and Awards:

President, Tropical Medicine Association of Washington, D. C.

Asst. Corresponding Secretary-Treasurer, Helminthological Society of Washington.

Member, Editorial Board, Experimental Parasitology

Chairman, NIAID Safety Committee

Member, American Society of Parasitologists Committee on Translations.

Member, Supervisory Committee NIH Credit Union

Publications:

McMaster, P. R. B., Powers, K. G., Finerty, J. F., and Schiffman, G.: Tolerance to Type III pneumococcal polysaccharide in monkeys. Immunol. Comm. 2: 361-370, 1973.

Garnham, P. C. C. and Powers, K. G.: Periodicity of infectivity of plasmodial gametocytes: The Hawking Phenomenon. Inter. J. Parasit. 4: 103-106, 1974.

Abdel-Wahab, M., Powers, K. G., Mohmoud, S. S., and Good, W. C.: Suppression of schistosome granuloma formation by malaria in mice. Am. J. Trop. Med. Hyg. (in press), 1974.

Warren, M., Powers, K. G., Garnham, P. C. C. and Shiroishi, T.: Plasmodium cynomolgi: Influence of x-irradiation and sporozoite dilution on relapse patterns in infected rhesus monkeys. Exp. Parasit. (in press), 1974.

Serial No. NIAID 132-C
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Experimental Chemotherapy of Malaria and Other Protozoan Diseases.

Previous Serial Number: Same

Principal Investigator: Kendall G. Powers

Other Investigators: Masamichi Aikawa and Milford N. Lunde

Cooperating Units: Case Western Reserve University

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	8/12			8/12
Professional	5/12			5/12
Other	3/12			3/12

Project Description:

Objectives: To establish and maintain a series of models for use in evaluating potential antimalarial activity of various compounds. This includes parasites from birds, rodents and nonhuman primates. Drug activity is evaluated against both blood and tissue stages of the parasite. Compounds are also tested against other protozoan diseases when promising drugs become available.

Methods Employed: Compounds with antimalarial activity are first tested orally and intraperitoneally in mice infected with Plasmodium berghei and in certain instances in mice infected with P. vinckei. Efficacy is based on the percentage of infected erythrocytes in treated mice as compared to untreated controls. Compounds also may by evaluated against strains of P. berghei resistant to chloroquine, pyrimethamine, quinine, DDS or certain long-acting sulfonamides and against P. vinckei resistant to pyrimethamine and chloroquine. When activity warrants, compounds are evaluated against

blood- and sporozoite-induced P. gallinaceum in chickens and against P. cynomolgi, P. Knowlesi and/or P. coatneyi in rhesus monkeys.

An additional host-parasite system is currently being utilized to further evaluate compounds that have demonstrated antimalarial activity. Aotus trivirgatus, infected with chloroquine-resistant and normal strains of P. falciparum, are being used to study the mode of action and chemotherapeutic activity of active compounds in the search for drugs that are efficacious against chloroquine-resistant falciparum malaria in man.

In certain instances promising compounds or drug combinations are evaluated against Toxoplasma gondii in mice. Various drug regimens are administered at different times during the acute infection and the results are compared with drugs currently being used in the chemotherapy of human toxoplasmosis. Compounds found to be active against this stage are tested against cysts formed during the chronic stage of infection.

Major Findings: Clindamycin (U-21) and N-demethyl-4-pentyl clindamycin (U-24) have been shown to be curative against acute Toxoplasma gondii in mice. The antitoxoplasmic activity of these compounds was comparable to the pyrimethamine-sulfadiazine combination that is commonly used to treat this disease in humans. Since toxoplasmosis often becomes chronic, with cysts in various tissues and organs, these drugs were further tested against this stage of infection. Groups of mice were infected with T. gondii and about 3 months later, when large numbers of cysts were present throughout the body, they were treated for 8 weeks with a diet containing either U-21, U-24 or pyrimethamine-sulfadiazine. Sera were collected to detect antibodies specific for toxoplasma and tissues were injected from each mouse into clean mice to determine if infective cysts were present. More than 40 percent of the mice treated with U-21 or U-24 were found to be cured of their chronic infections. We also evaluated the effect of U-21, U-24 and the pyrimethamine-sulfadiazine combination against occular toxoplasmosis in mice. By subinoculation we determined that 25 percent of the mice in each of the treatment groups did not have toxoplasma organisms in the eyes. All of the eyes subinoculated from untreated mice were infected. Although the cure rate in chronically infected mice was quite low the results are encouraging, particularly since no drug is currently known to be effective against toxoplasma cysts.

The antimalarial activity and the mode of action of clindamycin (U-21) and N-demethyl-4-pentyl clindamycin (U-24) was studied in rhesus monkeys infected with Plasmodium knowlesi. This parasite has a 24 hour life cycle and produces a fulminating infection that usually terminates in death 3-6 days after patency. Both antibiotics were able to control the rapid rise in parasitemias within 48-60 hours when therapy was initiated early in the infection. However, parasitemias greater than 10 percent had to be treated initially with a more rapid acting antimalarial such as chloroquine or quinine before U-21 or U-24 could be used to cure the infection. Doses of 10 mg/kg of either antibiotic was found to be curative when given for 5 to 7 consecutive days. Parasites from monkeys treated with U-21 or U-24 were

studied by light and electron microscopy in order to determine morphologic changes produced by these drugs. By light microscopy the first indication of drug effect on the parasite was seen after about 24 hours. By 48 to 60 hours there were pronounced drug alterations as evidenced by dissociation of the cytoplasm from the chromatin material and by the general disruption and vacuolation of the cytoplasm. The cytoplasm also was less basophilic than in the normal parasites. The ultrastructural changes induced by the antibiotics were seen in all stages of the parasite but were particularly prominent in late trophozoites and schizonts. The mode of action appears to be primarily against the ribosomes and with time, about 24 to 48 hours, the ribosome appears to disintegrate producing amorphous, irregular areas in the cytoplasm. There appear to be secondary changes in the nucleus and mitochondria. Nuclei appear condensed and fibrilluar while the mitochondria appear enlarged with prominent DNA fibers. Lincomycin, the parent compound, has been reported to inhibit protein synthesis at the ribosome level in bacterial systems. Thus, our results in malarial parasites appear to correlate well with the mode of action of the parent compound. These studies indicate that the mode of action of clindamycin is different from that of the commonly used antimalarials such as the 4-aminoquinolines and the antifolic type compounds.

Significance to Biomedical Research and the Program of the Institute:

Certain strains of Plasmodium falciparum are resistant to chloroquine and most other synthetic antimalarials and there exists an urgent need for a safe, efficacious drug for the treatment of this disease. Considerable effort has been expended during the past 10 years to find such drugs and one of the more promising compounds in this respect is the antibiotic, clindamycin. This drug has been shown to possess a significant degree of antiparasitic activity against simian and human malaria and may prove useful in the treatment of resistant strains of falciparum malaria. Clindamycin is relatively nontoxic and is already commercially available for use in humans as an antibacterial agent. The curative activity of this drug against toxoplasmosis in mice suggests that this antibiotic also may be of value in the therapy of toxoplasmosis in humans, possibly even against infections acquired during pregnancy in which the use of sulfadiazine and pyrimethamine is questioned because of their toxocity.

Proposed Course of the Project: Preliminary studies in prisoner volunteers have demonstrated that clindamycin, in combination with quinine, cured three multidrug resistant strains of P. falciparum (Cambodian I, Burma (Thau) and Panama II) (see annual report 134-A). A field study is being proposed, in cooperation with the University of the Philippines, to study the efficacy of clindamycin against naturally acquired chloroquine-resistant falciparum malaria in human populations on Palawan Island, Philippines. Additional studies on the mode of action of clindamycin will be conducted in owl monkeys infected with P. falciparum. Radio-labelled clindamycin will be used to more precisely identify the exact site of action of the drug.

Serial No. NIAID 132-D
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on Drug Resistance in Experimental Malaria

Previous Serial Number: Same

Principal Investigator: Richard L. Jacobs

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	6/12			6/12
Professional	3/12			3/12
Other	3/12			3/12

Project Description:

Objectives: To investigate the mechanisms of acquired drug resistance in malarial parasites; to develop and characterize different drug-resistant strains of rodent malaria by cross-resistance patterns to various antimalarial drugs, pathogenicity, morphological changes, and stability of acquired resistance in the absence of selective drug pressure. To gain a better understanding of the mechanism of action of different antimalarial drugs.

Methods Employed: Various strains of Plasmodium berghei are maintained in mice by blood transfer. Gametocyte-producing strains are maintained by cyclical passage through Anopheles stephensi, A/J mice, and hamsters. Standard dose-suppression tests are conducted to establish the sensitivity of the parent strain of P. berghei to the various drugs under study prior to selection for resistant strains. Parasitemia is followed in blood smears stained by the Giemsa procedure. The suppressive effect of different doses of drug is determined by comparison of the mean percent parasitemia in treated groups with the untreated control group of mice.

The procedure used to develop drug-resistant strains of P. berghei was described in the previous report. In brief, it involves repeated blood passages of the parasite in groups of mice treated with the highest dose of drug that allows for some development of during each passage. Transfer to fresh mice is usually made by day 7, using large numbers of parasitized erythrocytes (10 million/mouse) which are inoculated intravenously. Drugs are administered orally (by stomach tube) as single daily doses on days 0, 1, 2, and 3 after inoculation of parasites. Drug dosages are increased as indicated by the degree of parasite development in the previous passage in order to maintain a maximum amount of selective drug pressure. Parasite strains are considered totally resistant when they develop in mice treated with the maximum tolerated dose (MTD) of drug. Totally resistant strains are subsequently maintained in mice treated with the MTD of drug during each passage. In order to assess the stability of resistance in the absence of selective drug pressure, the resistant strain is also subpassaged repeatedly in untreated mice and tested periodically for drug sensitivity. Selected samples of infected blood are preserved at -70°C. during the development of resistance for future reference.

Major Findings: The development of resistance to the semisynthetic antibiotics clindamycin and minocycline by P. berghei has been demonstrated. However, resistance to these compounds developed less readily than resistance to the more commonly used antimalarials such as chloroquine, quinine, pyrimethamine or primaquine. The development of resistance to one antibiotic does not confer cross-resistance to the other or cross-resistance to the other antimalarial drugs tested to date.

P. berghei became totally resistant to clindamycin during 42 successively treated passages in mice over a period of 294 days. This represents approximately a 40-fold increase in resistance since the parent strain did not tolerate dosages greater than 25 mg/kg/day and the resistant strain develops in mice treated with 1000 mg clindamycin which is the MTD for mice. Total resistance to minocycline by P. berghei has not been attained. The parent strain failed to develop in mice treated with doses of minocycline greater than 100 mg/kg/day. After 86 successive minocycline-treated passages over a period of about 600 days, the strain tolerated daily doses of 600 mg/kg/day. This is therefore less than total resistance to minocycline as mice tolerate doses up to 1000 mg/day, but does represent a 6-fold increase in degree of resistance to minocycline.

Neither the clindamycin- nor minocycline-resistant strains of P. berghei show evidence of cross-resistance to such commonly used antimalarials as chloroquine, quinine or pyrimethamine. Resistance to both antibiotics appear to be reasonably stable in the absence of selective drug pressure. The clindamycin-resistant strain failed to revert to normal sensitivity during 51 successive untreated passages in mice and resistance to monocycline has persisted through 15 untreated passages. The development of resistance to clindamycin or to minocycline resulted in no gross evidence of changes in morphology of the parasite or in virulence (rate of growth). In each case, pigment production by the parasite appears to be normal. Development of a high degree resistance to both clindamycin and minocycline has not been

accomplished during 15 clindamycin-treated passages of the minocycline-resistant strain.

Significance to Biomedical Research and the Program of the Institute:

Drug-resistant strains of malaria, particularly resistance to chloroquine, continue to present a world-wide problem in the chemotherapy of malaria. Strains of P. falciparum that are resistant to all of the commonly used synthetic antimalarials, as well as to quinine, continue to be encountered in widely separated areas of the world. Also, other species of malaria that infect man may become refractory to presently effective drugs. The appearance of drug-resistance in malarial parasites of man has served to emphasize the need for a better understanding of the basic mechanisms of drug action on the parasite and other aspects of host-drug-parasite relationships. Plasmodium berghei has proven to be a valuable laboratory model for the investigation of the mode of action of various antimalarial compounds and some of the basic aspects of acquired drug resistance in malarial parasites.

Recent reports regarding the successful use of antibiotics, especially derivatives of lincomycin and tetracycline, for the treatment of drug-resistant malaria in man raises questions regarding the prolonged usefulness of these compounds for this purpose. If no cross resistance occurs, then resistance to one will not invalidate the usefulness of the other compound. Studies in mice infected with P. berghei should give information regarding the probability of development of resistance to these compounds by malarial parasites, cross-resistance patterns, and the stability of resistance in the absence of selective drug pressure. These results should be of value in predicting the usefulness of the antibiotic derivatives for the treatment of malaria in man.

Proposed Course of the Project: The study will be continued with emphasis on explaining the mechanism(s) by which parasites become resistant to drugs. Using radio-labelled clindamycin, differences in uptake of the drug between sensitive and resistant strains will be investigated.

Serial No. NIAID 132-E
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on the Labeling of Sporozoites of Malaria

Previous Serial Number: Same

Principal Investigators: Richard L. Jacobs and Louis H. Miller

Other Investigators: Robert W. Gwadz and Louis C. Koontz

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	20/12			20/12
Professional	11/12			11/12
Other	9/12			9/12

Project Description:

Objectives: To develop methods for labeling sporozoites in order to determine their distribution in the mammalian host (kinetics) and to follow development of the sporozoite through the exoerythrocytic stages within the parenchymal cells of the liver. The effect of drugs and immunity on the kinetics of sporozoite distribution and the developing EE forms will be investigated. Labelled sporozoites will provide a useful tool for the study of relapse mechanisms.

Methods Employed: The NK-65 strain of Plasmodium berghei is maintained in cyclical passage through mosquitoes (Anopheles stephensi), A/J mice, and hamsters. P. cynomolgi is maintained in cyclical passage in rhesus monkeys and different species of mosquitoes (A. maculatus and A. freeborni). Sporozoites are harvested by dissection of infected mosquito salivary glands at appropriate times after the infective blood meal. Developing sporozoites are labelled in vivo by feeding appropriate radio-labelled compounds to infected mosquitoes. The degree of radioactive isotope incorporated into

sporozoites is determined by autoradiography. Viability of labelled sporozoites is assessed by intravenous injection into susceptible hosts. The development of the radioactive sporozoite in the mammalian host will be followed in tissue sections and in disassociated liver cells by autoradiography. The distribution of the labeled sporozoites will be studied by scintillation counting of tissue homogenates of various organs (extracts).

Major Findings: Procedures have been worked out for the radioactive labelling of developing sporozoites of P. berghei and P. cynomolgi using either of several tritiated purines (adenine, adenosine, deoxyadenosine, deoxyguanosine). Tritiated adenine fed to infected mosquitoes in syrup solution at levels of 5, 10 or 25 uCi/ml resulted in well labelled sporozoites with little apparent effect in their infectivity. The feeding of higher levels of tritiated adenine suppressed both sporozoite production and infectivity of sporozoites. The tritiated purines are incorporated in both nuclear and cytoplasmic regions of sporozoites. The degree of labelling, as evidenced by the numbers of grains associated with individual sporozoites on autoradiographs, was related to the level of isotope fed to mosquitoes. When low levels of isotope were fed, both the percentage of sporozoites with associated grains and the average degree of labelling can be increased by extending the time of exposure to the photographic emulsion--which ordinarily ranges from about 7 to 21 days.

Significance to Biomedical Research and the Program of the Institute:

The greatest gap in our knowledge of the life cycle of the malaria parasite involves the mechanism by which relapses occur. The generally accepted explanation involving the reinvasion of hepatic cells by merozoites liberated from the exoerythrocytic cycle is not proven. More recently, it has been suggested that relapses may result from some sporozoites that remain dormant for long periods of time within the hepatic parenchemal cells before developing into exoerythrocytic forms. To date, these latent forms have not been demonstrated, possibly because of their small size and similarity to the cytoplasm of liver cells in stained preparations. It is hoped that the present study will determine whether late EE forms in P. vivax and P. ovale are due to slow development from sporozoites of from invasion of merozoites from the primary EE form to the secondary EE forms. The results of this study should be of great importance in the chemotherapy of hepatic schizonts (causal prophylaxis and radical cure). In addition, radio-labelled sporozoites should be of value in sporozoite immunization studies to locate the site of antigenic stimulation.

Proposed Course of the Project: These studies will be continued with major emphasis on the location of labelled sporozoites in tissue and following the development of labelled sporozoites in the mammalian host, especially in monkeys infected with P. cynomolgi, in an attempt to demonstrate latent forms which may be responsible for relapses in this vivax-type malaria.

Publications:

Jacobs, R. L., Miller, L. H., and Koontz, L. C.: Labeling of malaria sporozoites (*Plasmodium berghei*) with tritrated purines. J. Parasit. (In press).

Serial No. NIAID 132-G
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Mononuclear phagocyte activity in malaria.

Previous Serial Number: None

Principal Investigator: David J. Wyler

Other Investigators: Louis H. Miller; John Gallin (LCI)

Cooperating Units: Southern Research Laboratories, Birminham, Alabama (Dr. Leon Schmidt)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	7/12			7/12
Professional	6/12			6/12
Other	1/12			1/12

Project Description:

Objectives: Histopathologic and physiologic observation's by ourselves and others implicate an important function for the spleen in host defense in malaria infection. Several lines of approach have been initiated to further investigate the role of the spleen specifically, and mononuclear phagocyte, generally in malaria.

Methods Employed: (1) Collaborates studies (with Dr. Schmidt) will collate existing data on the effect of splenectomy on host defense in simian malaria models. (2) Spleens from P. berghei infected mice are being studied for their content of a substance, not present in normal spleens which is chemotactic for monocytes. (3) Monocyte kinetics is being studied in the P. berghei-mouse model, particularly with respect to splenic localization of these cells.

Major Findings: (1) A chemotactic substance is present in malarious spleens of mice and rhesus monkeys at least as early as one week after infection, and disappears several weeks after treatment. (2) Monocytes rapidly accumulate in mouse spleen, doubling their number as early as 48 hours after infection.

Significance to Bio-medical Research and the Program of the Institute:

Malaria provides an excellent model for investigating splenic function in intravascular infections, an area that is so far poorly understood. Furthermore, the monocyte chemotactic substance found in malarious spleens may prove to represent the first in vivo model of a functional role for these substances, and may have broad implications in the mechanism of splenomegaly and recruitment of phagocytes in intravascular infections.

The study of monocyte kinetics in malaria may provide a system in which the relationship of the spleen to bone marrow function and circulating monocyte can be studied.

Proposed Course of the Project: In addition to investigating these problems, we eventually plan to study different monocyte populations in vitro with regard to phagocytic and killing functions of these cells in malaria.

Serial No. NIAID 132-H
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1973

Project Title: Studies of cell-mediated immunity (CMI) in human malaria.

Previous Serial Number: 133-E

Principal Investigator: David J. Wyler

Other Investigators: Richard Smith (NCI), Robert Tigelaar (LMI), Leonard Altman (NIDR) and Louis H. Miller

Cooperating Units: University of Maryland (Dr. David Clyde); Maryland House of Correction (Jessup); Howard University Student Health Services (Dr. Samuel McCottry); District of Columbia Veterans Administration Hospital (Dr. Richard Knauft).

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	7/12			7/12
Professional	6/12			6/12
Other	1/12			1/12

Project Description:

Objectives: To continue investigations into various aspects of cell-mediated immunity in patients with previous malaria infections.

Methods Employed: Peripheral blood lymphocytes from patients are cultured in the presence of an antigen preparation from erythrocytes infected with P. falciparum, and proliferative response and lymphokine production (MIF and CTX factor) are measured as in vitro correlates of CMI. The lymphocyte type (T or B cell) proliferating in response to antigen is

determined by post-culture rosetting of cells with appropriate markers or pre-culture separation of T and B cells to provide a relatively pure starting population.

Attempts to determine if immunodepression at a cellular level occurs during patent malaria infection is underway, employing these techniques. Furthermore, preliminary studies have been initiated to determine if malaria parasites contain antigens cross-reacting with human HL-A antigens.

Major Findings: (1) Studies described in previous serial number NIAID 133-E (1972-73) were discontinued when extensive efforts to ellicit lymphoproliferative response or delayed hypersensitivity to malaria antigen in the rhesus monkey were inconsistent.

(2) Fifteen patients and nine controls were studied with in vitro methods and a specific and prominent lymphoproliferative response was ellicited in patients but not controls by lower concentrations of malaria antigen. A non-specific proliferative response, probably representing mitogenic activity, was noted with higher concentrations of antigen in controls and cord-blood lymphocytes as well. Evidence to date suggests that both T and B cells are involved in proliferation.

(3) Preliminary studies suggest that a lympokine chemotactic for monocytes is specifically released from human lymphocytes cultured in the presence of malaria antigen.

Significance to Bio-medical Research and the Program of the Institute:

Recent developments in basic immunology can be applied to the study of malaria in a hope to better understand this complex disease, with an eye to eventual development of a vaccine.

Proposed Course of the Project: (1) Plans are underway for studying CMI in an endemic population with respect to the questions of immunosuppression during patent infection and characterization of CMI status of the population in several age groups. (2) Lymphokine production will be studied further. (3) The relation of HL-A genetics and response to the non-specific response in normals will be studied.

Publications:

Wyler, D.J. and Oppenheim, J.J. Lymphocyte transformation in human Plasmodium falciparum malaria. J. Immunol. (in Press) 1974.

Wyler, D.J. Lympocyte transformation in human falciparum malaria. Clin. Res., 22: 457A, 1974.

Serial No. NIAID 133
1. Parasitic Diseases
2. Malaria-Primate and Human Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Susceptibility of Anopheline Mosquitoes to Simian and Human Malaria Infections and Their Ability to Transmit to Simian Hosts

Previous Serial Number:

Principal Investigators: William E. Collins

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	57/12			57/12
Professional	6/12			6/12
Other	51/12			51/12

Project Description:

Objectives: (1) To determine the relative susceptibility of anopheline mosquitoes to simian and human malaria infection; (2) to determine the rate and degree of parasite development within various mosquito hosts; (3) to attempt transmission of the various simian malarias to a wide spectrum of simian hosts by the bites of infected mosquitoes.

Methods Employed: Isolates of simian malaria are maintained either in monkey hosts or in the frozen state for use in transmission studies. Various species of Anopheles mosquitoes are allowed to feed on simian hosts infected with Plasmodium brasilianum, P. cynomolgi, P. coatneyi, P. fieldi, P. simiovale, P. simium, P. fragile, P. gonderi, P. knowlesi, P. inui, P. falciparum, P. vivax and P. malariae. Infection potential to a given mosquito species of a given malaria strain in a variety of hosts is

determined and transmission is made by direct mosquito bites or by the intravenous inoculation of sporozoites.

Major Findings: During the year there were 284 inoculations of parasitized blood and 178 inoculations of viable sporozoites into 462 monkeys. Of these, 380 resulted in patent parasitemias. A total of 2,687 lots of mosquitoes were fed on these animals. 54,868 mosquitoes were dissected and examined for the presence of oocysts on the guts and 785 for the presence of sporozoites in the salivary glands. Sporozoite transmission was obtained to 114 monkeys which included P. cynomolgi, P. fragile, P. knowlesi, P. coatneyi, P. gonderi, P. inui, P. vivax and P. falciparum. Transmissions were obtained using Anopheles freeborni, A. maculatus, A. b. balabacensis and A. albimanus mosquitoes.

The Aotus trivirgatus monkey was shown to be susceptible to P. fragile, a parasite biologically similar to P. falciparum and to P. knowlesi via mosquito feeding. Plasmodium cynomolgi infections were established by the inoculation of parasitized blood. Other simian malarias, P. coatneyi, P. fieldi, and P. gonderi could not be transmitted to this host.

Infection of mosquitoes on recurring peaks in the parasitemias of P. vivax in Aotus monkeys indicated that sporozoite induced infections would more readily infect mosquitoes than would blood induced and that delayed splenectomy resulted in higher levels of mosquito infection than were obtained by feeding on intact or presplenectomized monkeys.

Significance to Biomedical Research and the Program of the Institute:

This project was part of an integrated program of the Laboratory of Parasitic Diseases in the study of simian malaria in monkeys and its relationship to man and human malaria. A clean-cut delineation of the parasite-vector relationship is essential for varied species of simian malaria, simulating all of the human species in life pattern and morphology, to carry on significant studies on the chemotherapy, immunology and other aspects of malaria research.

Proposed course of the Project: These studies have been terminated.

Publications:

Collins, W.E., Contacos, P.G., Stanfill, P.S., and Richardson, B.B.: Studies on human malaria in Aotus monkeys. I. Sporozoite transmission of Plasmodium vivax from El Salvador. J. Parasit., 59: 606-608, 1973.

Collins, W.E., Neva, F.A., Chaves-Carballo, E., Stanfill, P.S., and Richardson, B.B.: Studies on human malaria in Aotus monkeys. II. Establishment of a strain of Plasmodium falciparum from Panama. J. Parasit., 59: 609-612, 1973.

Collins, W.E., Miller, L.H., Glew, R.H., Contacos, P.G., Howard, W.A., and Wyler, D. J.: Transmission of three strains of Plasmodium falciparum from monkey to man. J. Parasit., 59: 855-858, 1973.

Collins, W.E., and Contacos, P.G.: Malaria relapse mechanism. Trans. Roy. Soc. Trop. Med. Hyg. 67: 617-618, 1973.

Collins, W.E., Stanfill, P.S., Skinner, J.C., Harrison, A.J., and Smith, C.S.: Studies on human malaria in Aotus monkeys. IV. Development of Plasmodium falciparum in two subspecies of Aotus Trivirgatus. J. Parasit. (In Press).

Collins, W.E., Skinner, J.C., Richardson, B.B., Stanfill, P.S., and Contacos, P.G.: Studies on human malaria in Aotus monkeys. V. Blood-induced infections of Plasmodium vivax. J. Parasit. (In Press).

Collins, W.E. and Contacos, P.G.: Observations of the relapse activity of Plasmodium simiovale in the rhesus monkey. J. Parasit. (In Press).

Collins, W.E., Stanfill, P.S., Richardson, B.B., and Smith, C.S.: Transmission of Plasmodium fragile to Aotus trivirgatus monkeys. J. Parasit. (In Press).

Omar, M.S. and Collins, W.E.: The antimalarial activity of RC-12 against gametocytes and sporogony of Plasmodium cynomolgi. Trans. Roy. Soc. Trop. Med. Hyg. 67: 423-424, 1973.

Omar, M.S., Collins, W.E. and Contacos, P.G.: Gametocytocidal and sporontocidal effects of antimalarial drugs on malaria parasites. I. Effects of single and multiple doses of primaquine on Plasmodium cynomolgi. Exp. Parasitol. 34: 229-241, 1973.

Omar, M.S., Collins, W.E., and Contacos, P.G.: Gametocytocidal and sporontocidal effects of antimalarial drugs on malaria parasites. II. The action of the folic reductase inhibitors, chloroguanide and pyrimethamine against Plasmodium cynomolgi. Exp. Parasitol. (In press).

Omar, M.S. and Collins, W.E.: Studies on the antimalarial effects of RC-12 and WR 14,997 on the development of Plasmodium cynomolgi in mosquitoes and rhesus monkeys. Am. J. Trop. Med. Hyg. 23: 339-349, 1973.

Serial No. NIAID 133-A
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies of the Exoerythrocytic Stages of Primate Malaria

Previous Serial Number: NIAID 133-A

Principal Investigators: William E. Collins

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	23/12			23/12
Professional	3/12			3/12
Other	20/12			20/12

Project Description:

Objectives: (1) To describe the exoerythrocytic (EE) stages of species and strains of malaria in which they have not previously been described; (2) to study the histochemical reaction of EE bodies; (3) to study the prophylactic and curative effects of antimalarial drugs on the EE stages of malaria parasites and to determine the morphologic alterations within the EE stages due to antimalarial drugs.

Methods Employed: Exoerythrocytic forms are produced in a new host by transmission through a mosquito vector from a previously infected host. This is accomplished: (1) by mosquito bite; (2) by direct inoculation of the sporozoites into the liver; or (3) by intravenous inoculation of sporozoites.

Livers are biopsied on selected days after exposure or following drug therapy. Biopsies are processed after paraffin imbedding and stained with Giemsa.

Major Findings: Thirty-seven liver biopsies were made in two species of non-human primates in attempts to find ezoerythrocytic (EE) stages of five species of malaria parasites. Sixteen of these were shown to contain EE bodies.

A concerted effort (21 biopsies) was made to find and describe the EE stages of P. fragile. Seven 8, 9 and 10 day forms were found. Additional samples of the 8-day EE forms of P. vivax in the Aotus monkey were also found.

Proposed Course of the Project: This project has been terminated.

Publications:

Collins, W.E., Contacos, P.G., Jumper, J.R., Smith, C.S., and Skinner, J.C.: Studies on the exoerythrocytic stages of simian malaria. VIII. Plasmodium knowlesi. J. Parasit. 59: 344-352, 1973.

Krotoski, W.A., Collins, W.E. and Jumper, J.R.: Detection of early schizonts of Plasmodium cynomolgi by immunofluorescence. Am. J. Trop. Med. Hyg. 22: 443-451, 1973.

Collins, W.E., Contacos, P.G., Jumper, J.R., Smith, C.S., and Skinner, J.C.: Studies on human malaria in Aotus monkeys. III. Exoerythrocytic stages of the Salvador II strain of Plasmodium vivax. J. Parasit. 59: 859-866, 1973.

Serial No. NIAID 133-B
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Reports
July 1, 1973 through June 30, 1974

Project Title: Immuno-ecology of Primate Malaria

Previous Serial Number: Same

Principal Investigators: William E. Collins

Other Investigators: None

Cooperating Units: Central America Malaria Research Station, El Salvador

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned To project	Guest Workers	Total
Total	13/12			13/12
Professional	3/12			3/12
Other	10/12			10/12

Project Description:

Objectives: To explore the total ecology of malaria and to correlate circulating antibodies with the presence of parasites and to orient both of these factors with transmission cycles, level of endemicity, and clinical disease.

Methods Employed: Blood samples, on filter paper, are collected from human populations in areas endemic for malaria. These specimens are mailed back to the central laboratory in Chamblee, Georgia for determination of fluorescent antibody titers. The accompanying field studies vary in scope, but wherever possible the following investigations are carried out: (1) Giemsa-stained blood films of the human population for parasite rates and species identification; (2) surveys for malaria parasites in mammals other than man; and (3) studies on the anopheline fauna, including identification, seasonal distribution, biology and malaria infection rate.

Serial No. NIAID 133-B

Major Findings: Project terminated.

Proposed Course of the Project: Terminated.

Serial No. NIAID 133-C
1. Parasitic Diseases
2. Primate & Human Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Fluorescent Antibody Studies in Simian and Human Malaria

Previous Serial Number: Same

Principal Investigator: William E. Collins

Other Investigators: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	3/12			3/12
Professional	1/12			1/12
Other	2/12			2/12

Project Description:

Objectives: (1) To determine the development and long-term persistence of specific antibody in malaria-infected humans and monkeys; (2) to study the immunologic relationships between the different species of human and simian malarias; and (3) to determine the effect of biological and surgical immunosuppression on the development and persistence of antibody reactions.

Methods Employed: Serum samples are taken from patients and animals prior to infection and at regular intervals thereafter to determine by fluorescent antibody methods the rate of specific antibody production and the persistence and titer of antibody during the months and years following termination of the malaria infection. Animals having past malarial infection are challenged with heterologous species and strains of Plasmodium sp. and the immunologic response studied by the fluorescent antibody technique. Animals are given curative and subcurative treatments with antimalarial drugs and the effect measured using the FA technique. Animals splenectomized or otherwise surgically treated before and after

infection are studied in regard to the FA response. Sera from patients infected with malaria are studied using the specifically labeled immune globulins to determine the patterns of response to each.

Major Findings: Project terminated

Proposed Course of the Project: Terminated.

Serial No. NIAID 133-D
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Immunological Studies on Primate Malaria

Previous Serial Number: 133-D

Principal Investigators: William E. Collins

Other Investigators: None

Cooperating Units: Dr. Peter G. Contacos, Center for Disease Control, Atlanta, Georgia

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	26/12			26/12
Professional	2/12			2/12
Other	24/12			24/12

Project Description:

Objectives: (1) To evaluate the possibilities for effective biologic prophylaxis against malaria; (2) to analyze the antigenic composition of various species and/or strains of simian malaria; and (3) to localize (insofar as possible) the morphological and/or antigenic unit(s) principally responsible for specific immunofluorescent reactions following naturally acquired malarial infections.

Methods Employed: For the antigenic analysis and the active immunization studies, purified parasite suspensions are obtained from monkeys at the time of peak parasitemia. Monkeys are actively immunized with protein extracts, with intact parasitized erythrocytes, or with intact parasites freed from the red blood cell by lysis, with fractions of hemolysates from parasitized erythrocytes, or with residue of extracted ruptured parasites. After a suitable period of time, the monkeys are challenged with malaria. Presence of antibody in the serum of the

immunized animals is confirmed by indirect FA and immunoelectrophoresis. Antigenic composition of the various preparations is studied by immunoelectrophoresis.

Major Findings: Studies were completed on the use of a heat stable (100°C) serum soluble antigen (SSA-S) of P. knowlesi for immunization of Macaca mulatta monkeys. Total antigen concentrations of less than 10 mg. or greater than 40 mg. were ineffective. Although immunization resulted in increased survival rates and reduced peak parasitemias, the results were inconsistent. This antigen, by itself, appears unsatisfactory.

Studies were made on the transfer of spleen cells from monkeys immune to P. knowlesi to normal animals. The mortality rate and mean peak parasitemias were higher in these animals (8) than in control monkeys inoculated with a like number of cells from normal uninfected monkeys.

Proposed Course of the Project: The studies have been terminated.

Serial No. NIAID 133-E
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Study of Cell-mediated Immunity (CMI) in Simian Malaria

Previous Serial Number: Same

Principal Investigator: Dr. David J. Wyler; Dr. Wyler transferred from Chamblee, Georgia to Bethesda, Md.

Please see Project No. NIAID 132-H

Serial No. NIAID 133-F
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The role of malaria in the induction of lymphoma by Epstein-Barr virus (EBV).

Previous Serial Number: Same

Principal Investigators: Franklin A. Neva, Louis H. Miller, and David J. Wyler

Other Investigators: William E. Collins

Cooperating Units: M. A. Epstein, Department of Pathology, University of Bristol, Bristol, England

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	2/12			2/12
Professional	1/12			1/12
Other	1/12			1/12

Project Description:

Objectives: (1) To determine whether lymphoma can be induced in monkeys by inoculation with EBV and malaria parasites; (2) to determine what role malaria plays in the oncogenesis of Burkitt's lymphoma; (3) to establish an *in vitro* culture line of any tumor that so arises.

Methods Employed: Three groups of owl monkeys (*Aotus trivirgatus*) were inoculated with EBV-containing lymphoblastoid cells, either on the same day as, or nine or twenty-five days following blood-induced infection with *Plasmodium simium*. Baseline and multiple post-inoculation serum specimens were obtained for determination of titers of anti-EBV antibodies. Daily parasite counts were made.

Major Findings: Since inception of this study, it has been subsequently observed that none of the monkeys inoculated with EBV become sero-positive for the virus.

Proposed Course of the Project: This project has been terminated.

Serial No. NIAID 133-G
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on Gametogenesis and the Infectivity of Primate Malarias to Mosquitoes

Previous Serial Number: 133-G

Principal Investigators: Robert W. Gwadz

Other Investigators: William E. Collins

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	16/12			16/12
Professional	6/12			6/12
Other	10/12			10/12

Project Description:

Objectives: To determine factors which regulate gametogenesis and viability of gametes in various human and simian malarias.

Methods Employed: The viability and infectivity of Plasmodium sp. are being studied in various anopheline mosquitoes and in vitro with particular reference to "gamete-specific" host immunity. A membrane blood-feeding system is being utilized to measure the effects of various blood components on the infectivity of gametocytes to mosquitoes.

Plasmodium falciparum and P. vivax infections in Aotus monkeys usually produce non-infectious gametocytes in spite of continually increasing parasitemias and gametocytemias; consequently, studies of these parasites requiring sporozoite induced infections have been difficult or impossible.

We are currently employing the P. cynomolgi rhesus monkey model system to study those factors which inhibit or enhance the infectivity of

gametocytes.

Major Findings: Gametocyte infectivity has been significantly increased by various treatments of parasitized blood prior to feeding mosquitoes through membranes. A 5-fold increase in mosquitoes infections has been achieved. Parasitized blood cells suspended in a defined medium are also highly infectious.

Significance to Biomedical Research and the Program of the Institute:

The full utilization of the human malaria/Aotus monkey models for research has been hampered by the frequent non-infectivity of gametocytes to mosquitoes. Improvement of gametocyte infectivity would significantly enhance the value of these important model systems.

An understanding of "gamete-specific" immunity in malaria should provide insights into some of the basic mechanisms of host immunity and cycles of host infectivity. "Gamete-specific" immunization may also have potential for interruption of transmission in malarious areas.

Proposed Course of the Project: This project will continue and be expanded in order to define those factors which enhance or suppress gamete infectivity. The role of the spleen in "gamete-specific" immunity and circadian rhythms of gamete infectivity will be explored. The feasibility of immunization against gametes will be investigated.

Serial No. NIAID 133-H
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Effects of Chemosterilization on the Development and Transmission of Malaria Parasites in Mosquitoes.

Previous Serial Number: 133-H

Principal Investigators: Mohamed S. Omar and William E. Collins

Other Investigators: Robert W. Gwadz

Cooperating Units: Entomology Research Division, Agriculture Research Service, USDA, Gainesville, Florida

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	12/12			12/12
Professional	5/12			5/12
Other	7/12			7/12

Project Description:

Objectives: To study the effects of chemosterilants on the development of malaria parasites in various anopheline vectors and to determine the rate of malaria transmission by chemosterilized mosquitoes.

Methods Employed: Various malaria parasites are used in this study including the simian parasites, Plasmodium cynomolgi, P. gonderi in rhesus monkeys; the human malaria parasite, P. vivax in owl monkeys. Sterilized mosquitoes used for transmission studies are Anopheles freeborni, A. quadrimaculatus, A. maculatus and A. albimanus. Mosquitoes were sexually sterilized by dipping pupae for 1 to 4 hours in 1% aqueous solution of the chemosterilant p, p,-bis (1-aziridinyl)-N-methyl phosphirothioic amide (ENT-61585). Pupae are then removed from the sterilizing solution and rinsed with water before being placed in emergency containers. Adult mosquitoes were fed on monkeys infected with P. vivax and examined for

subsequent parasite development.

Major Findings: Anopheles albimanus and A. freeborni responded differently to pupal treatment and subsequent adult infection. Virtual complete sterility of A. albimanus and A. freeborni was obtained after 1- and 4-hour exposures respectively. The difference in susceptibility between sterilized and normal mosquitoes to infection with P. vivax was more pronounced in A. albimanus than in A. freeborni. The overall infection rate of treated A. albimanus was significantly reduced over that of control mosquitoes. The infection in sterilized mosquitoes was not eliminated and sporogony of P. vivax was completed with motile sporozoites in the salivary glands.

Significance to Biomedical Research and the Program of the Institute:

One of the promising aspects in bological control of mosquitoes is the genetic manipulation of insect populations. One of these approaches is the sterile-male release technique involving sterilization and release of laboratory-bred males to compete with wild males in the field for mating with female mosquitoes. Sterilization of mosquitoes could be achieved either by X-radiation or by the use of chemosterilants. If the chemosterilant used for treating males could also be used at the appropriate dose level to sterilize female mosquitoes and likewise disrupt or prevent the development of malaria parasites within the mosquito host, its impact would have obvious advantage in genetic control operations. Thus, instead of the exclusive release of sterilized males, sexually sterilized females could also be released in the field without the potential hazards of increasing the incidence of malaria transmission.

Proposed Course of the Project: This project has been terminated.

Publications:

Omar, M.S., Collins, W.E. and Gwadz, R.W.: Development of Plasmodium vivax in chemosterilized anopheline mosquitoes. Am. J. Trop. Med. Hyg. 23: 334-338, 1974.

Serial No. NIAID 133-I
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on the Genetics of Vector Capacity of Anopheline Mosquitoes for Primate Malarias.

Previous Serial Number: 133-K

Principal Investigators: Robert W. Gwadz

Other Invesitgators: William E. Collins

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	15/12			15/12
Professional	5/12			5/12
Other	10/12			10/12

Project Description:

Objectives: (1) To select lines of mosquitoes susceptible or refractory to a particular species of primate malaria; (2) to determine the mode of inheritance of susceptibility to this particular malaria; (3) to determine susceptibility of the selected mosquito line or lines to various geographic strains of this malaria and to other species of human and simian malaria; (4) to determine what physiologic factors may be involved in rendering certain mosquitoes refractory or susceptible to infection with human and simian malarias.

Methods Employed: Colonies have been established from individual females of Anopheles albimanus susceptible to the Gombok strain of Plasmodium cynomolgi. The mode of inheritance of susceptibility is being determined by standard methods of genetic analysis.

Major Findings: Initial attempts to select strains of A. albimanus susceptible to the human parasites, P. vivax and P. falciparum have been hampered by significantly reduced and unpredictable gametocyte infectivity in the Aotus monkey model. Consequently we tested a number of strains of P. cynomolgi (a vivax-like parasite of monkeys) for infectivity to A. albimanus and found the Gombok strain to be moderately infectious; all other strains of P. cynomolgi failed to infect A. albimanus. Selection of A. albimanus for susceptibility to the Gombok strain is underway.

Significance to Biomedical Research and the Program of the Institute:

Malaria is an entomological problem, but the genetic basis for vectorial capacity is poorly understood. Development of genetically defined lines of mosquito vectors will provide important tools for a variety of research efforts.

Susceptible and refractory lines will provide material for histochemical, physiological and bio-chemical investigations into the nature of the mosquito-malaria host-parasite relationship.

Refractory lines of mosquitoes should prove useful in biological control schemes involving vector displacement or genetic manipulation of vector populations.

Understanding of vector genetics will facilitate the analysis of populations of mosquitoes polymorphic with respect to vector capacity.

Proposed Course of the Project: These studies will be continued and expanded to include a number of anopheline species and their relationships with a variety of human and simian malarias.

Serial No. NIAID 133-L
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Role of Serum Complement in Primate Malaria

Previous Serial Number: Same

Principal Investigator: Richard H. Glew

Other Investigators: William E. Collins

Cooperating Units: John Atkinson and Michael M. Frank, Laboratory of Clinical Investigation

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	3/12			3/12
Professional	2/12			2/12
Other	1/12			1/12

Project Description:

Objectives: This study was designed to determine levels of serum complement (generally as C'4) during the course of infection in rhesus monkeys with Plasmodium coatneyi. In addition, an attempt was made to evaluate the possible protective role of serum complement in the host defenses against malaria by depleting the late components of serum complement through injections of cobra venom factor into the monkeys.

Proposed Course of the Project: Terminated (early in year because of closing of Chamblee Unit).

Serial No. NIAID 133-M
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Induction of Quinine Resistance in P. falciparum in Aotus Monkeys

Previous Serial Number: Same

Principal Investigator: Richard H. Glew

Other Investigators: Louis H. Miller and William E. Collins

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	5/12			5/12
Professional	2/12			2/12
Other	3/12			3/12

Project Description:

Objectives: Experimental induction of quinine resistance in plasmodia has been accomplished only in P. berghei (in mice) and P. relictum (in canaries). However, studies in human volunteers by this Unit suggested that the Colombia (KSN) strain of P. falciparum acquired decreased sensitivity to quinine during sequential courses of increasing days of quinine therapy. Accordingly, this study was instituted in an attempt to induce increasing quinine resistance in P. falciparum in Aotus monkeys.

Proposed Course of the Project: Terminated. (early in year because of closing of Chamblee Unit).

Serial No. NIAID 134
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Pathogenesis of the malarial paroxysm

Previous Serial Number: Same

Principal Investigator: Franklin A. Neva

Other Investigators: Michael Frank, M.D.; John Atkinson, M.D.; and Louis H. Miller, M.D.

Cooperating Units: Laboratory of Clinical Investigation, Section on Medical Parasitology, NIAID

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	4/12			4/12
Professional	2/12			2/12
Other	2/12			2/12

Project Description:

Objectives: (1) To define, with greater precision than has been done previously, the time relationshio of parasite development to onset and subsequent elements of the malarial paroxysm and (2) to determine causative factors in the genesis of fever and other manifestations of the malarial paroxysm.

Methods Employed: Study of the time relationship of parasite development to the onset and subsequent elements of the malarial paroxysm is limited to patients with naturally occurring or induced malaria whose infections exhibit a highly synchronized cycle of parasite development and/or a well synchronized cycle of clinical signs and symptoms. Thin blood

smears are prepared throughout a selected paroxysm at 15 to 30 minutes intervals while periodic blood specimens are taken for hematologic and serologic determinations, and body temperature is monitored. Subsequently, several observers independently perform parasite differential counts on each slide by recording total numbers of specified parasite forms observed per 80,000 RBC's.

Serum complement levels, assayed with a standardized 50% hemolytic endpoint technique, are measured at slected intervals early after the onset of patent malaria as well as late in the illness prior to treatment. When possible, a paroxysm during the primary attack as well as during a relapse is studied. Special effort is made to bracket well-defined fever paroxysms with complement determinations and correlate the serum complement levels with the immune status of the host, as measured by the IFA and CF techniques, and the parasite density.

Attempts to demonstrate a transferable pyrogen and/or other biologically active substances involves withdrawal of 250 or 500 ml of blood at a specified time during a well-synchronized paroxysm. The blood, withdrawn and handled using standard blood banking procedures, is re-infused in the same patient as whole blood or component fractions after the patient has been treated.

Major Findings: (1) The study on alterations in serum complement and in complement components during paroxysms of P. vivax malaria was submitted for publication. Some additional work since last year's report revealed that the consumption of C' involved only or mainly early components (C1, C4 and C2) with much less or no change in late components (C3 and C6). These findings parallel those occurring in the rhesus monkey with P. coatneyi malaria. (2) Correlation of the time-course of schizogony with fever and other events of the malarial paroxysm has not yet been completed. Some of the data has been tabulated and it appears that sufficient material from suitable cases is available to complete the analysis.

Significance to Biomedical Research and the Program of the Institute:

From work of others being submitted for publication and in progress it is apparent that there is considerable interest in the role of complement in malaria. Our studies which established basic factors controlling C' changes and the overall pattern will be useful as a basis for future work. The observations on time-course of schizogony in relation to other events of the paroxysm have been helpful in establishing exactly when specimens should be taken in efforts to demonstrate pyrogens or other mediators of fever.

Proposed Course of Project: Some of the specimens which we have from this study could probably be used to complete some aspects of the study, such as demonstration of circulating antigen during the paroxysm. Also, complement utilization in-vitro may be investigated as well as selected patients at the Clinical Center.

Publications:

Neva, F.A., Howard, W.A., Glew, R.H., Krotoski, W.A., Gam, A.A., Collins, W.E., Atkinson, J.P. and Frank, M.M. Relationship of serum complement levels to events of the malarial paroxysm. J. Clin. Invest. (in Press).

Serial No. NIAID 134-A
1. Parasitic Diseases
2. Human Malaria
3. Atlanta, Georgia

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Evaluation of Antimalarial Drugs and/or Drug Regimens

Previous Serial Number: Same

Principal Investigators: Louis H. Miller and David J. Wyler

Other Investigators: William E. Collins and Franklin A. Neva

Cooperating Units: Department of Justice, Bureau of Prisons

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	18/12			18/12
Professional	2/12			2/12
Other	16/12			16/12

Project Description:

Objectives: To evaluate standard antimalarial agents as well as new drugs with demonstrated antimalarial activity, and to develop acceptable regimens for their use in the treatment of human malaria.

Methods Employed: Healthy inmate volunteers at the U.S. Penitentiary, Atlanta, Georgia, are fully informed of the risks and discomforts of malaria, and are not subjected to coercion or excessive inducements. Volunteers are under the 24-hour care and supervision of the residence investigators. For the evaluation of chemotherapeutic agents, volunteers are exposed to the bites of several mosquitoes infected with malaria. Treatment is begun after patent parasitemia is demonstrated. Volunteers are followed with daily thick blood smears for at least 3 to 6 months after treatment.

Major Findings: The efficacy of clindamycin, a slow acting antimalarial, in combination with a rapid acting schizonticidal drug, quinine, was determined against 3 multidrug resistant strains of Plasmodium falciparum (Cambodian I, Burma (Thau) and Panama II). No recrudescences occurred after treatment with quinine 650 mg orally every 8 hours for 3 days and clindamycin 450 mg orally every 6 hours for 3 days, when administered either simultaneously or sequentially to 13 volunteers. Quinine alone for 3 days was not curative against infections with these strains. During a 3 day course of therapy, blood levels of quinine or clindamycin were not influenced by administration of the other drug.

Significance to Bio-medical Research and the Program of the Institute:

The widespread occurrence of multi-drug resistant Plasmodium falciparum in recent years has stimulated interest in the search for additional chemotherapeutic methods and drugs against malaria. Our studies thus far have shown that clindamycin, in combination with quinine, produced radical cure when tested against multi-drug resistant P. falciparum from Asia and Central America. Clindamycin appears to have an advantage over other antibiotics tested to date (tetracycline and minocycline) in that clindamycin may require a shorter course of therapy and offers another compound against drug resistant falciparum malaria.

Proposed Course of the Project: The studies have been terminated with the closing of the Federal Penitentiary, Atlanta, Georgia. The efficacy of clindamycin to date warrants further studies of this drug in other malaria research units and in the field

Publications:

Miller, L.H., Glew, R.H., Wyler, D.J., Howard, W.A., Collins, W.E., Contacos, P.G., and Neva, F.A. Evaluation of clindamycin in combination with quinine against multidrug resistant strains of Plasmodium falciparum. Amer. J. Trop. Med. Hyg. (in Press), 1974.

Glew, R.H., Miller, L.H., Collins, W.E., Howard, W.A., Wyler, D.J., Chaves-Carballo, E., and Neva, F.A. Response to treatment in man of multidrug resistant Plasmodium falciparum from Panama. Amer. J. Trop. Med. Hyg. 23: 1-7, 1974.

Glew, R.H., Briesch, P.E., Krotoski, W.A., Contacos, P.G., and Neva, F.A. Multidrug resistant Plasmodium falciparum from eastern Colombia. (in Press), 1974. J. Infect. Dis.

Miller, L.H., Wyler, D.J., Glew, R.H., Collins, W.E., and Contacos, P.G. Sensitivity of four Central American strains of Plasmodium vivax to primaquine. Amer. J. Trop. Med. Hyg. (in Press), 1974.

Miller, L.H. Transfusion malaria, in Transmissible Disease and Blood Transfusion. Jamieson and Greenwalt (ed.)

Contacos, P.G., Coatney, G.R., Collins, W.E., Briesch, P.E., and Jeter, M.H.: Five day primaquine therapy--an evaluation of radical curative activity against vivax malaria infection. Am. J. Trop. Med. Hyg. 22: 693-695, 1973.

Contacos, P.G., Collins, W.E., Chin, W., Jeter, M.H., and Briesch, P.E.: Combined chloroquine--primaquine therapy against vivax malaria. Am. J. Trop. Med. Hyg. 23: 310-312, 1973.

Collins, W.E., Contacos, P.G., and Chin, W.: Experimental infection in man with Plasmodium malariae. Am. J. Trop. Med. Hyg. 22: 685-692, 1973.

Serial No. NIAID 67C

1. Laboratory of Parasitic Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on arboviruses other than dengue

Previous Serial Number: Same

Principal Investigator: Dr. Robert Tesh

Other Investigators: Dr. Leon Rosen, Dr. Duane Gubler

Man Years:

	NIAID Staff
Total:	12/12
Professional:	12/12
Other:	0/12

Cooperating Units: Department of Tropical Medicine and Medical Microbiology, University of Hawaii School of Medicine, Honolulu; Middle America Research Unit - Gorgas Memorial Laboratory, Ancon, Canal Zone; Yale Arbovirus Research Unit, Yale University School of Medicine, New Haven, Conn.; U.S. Naval Medical Research Unit No. 2, Taipei, Taiwan; University of California Center for Medical Research, University of Malaya, Kuala Lumpur, Malaysia; Study of Child Growth and Development and Disease Patterns in Primitive Cultures, NINDS, NIH; U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Md.

Project Description:

Objectives

1. To study the epidemiology of arthropod-borne virus infections other than dengue of public health importance on the Pacific islands and in Southeast Asia.

2. To study the genetics of susceptibility and resistance to Chikungunya virus infection in selected geographic strains of *Aedes albopictus*.

3. To elucidate the epidemiology of sandfly fever through serologic and experimental studies of viruses comprising the Phlebotomus fever group.

Methods Employed

Studies of experimental arbovirus infection in mosquitoes after intrathoracic inoculation or feeding were done utilizing laboratory colonies of Aedes albopictus and Culex fatigans. Culture and titration of insect specimens were done in tube or microplate cultures of LLC-MK_2 and Vero cells. Plaque neutralization studies of human sera were performed in microplate cultures of LLC-MK_2 or Vero Cells.

Major Findings

Mosquito genetics studies with Chikungunya virus

Using Chikungunya as a model of arbovirus infection, 11 different geographic strains of A. albopictus were fed a standard virus dose. Susceptibility of the various strains to Chikungunya oral infection varied from 10 to 80%. Six strains were subsequently chosen and inbred by sibling matings for several generations in an attempt to select out resistant and susceptible lines. Despite intensive inbreeding of offspring from infected or non-infected females, it has not been possible to select out purely resistant or susceptible lines. In fact, the percentage of infected females in each subsequent inbred generation has shown little variation from the susceptibility of the original parent stocks. This suggests that either susceptibility and resistance to oral Chikungunya infection is not genetically determined or that the genetic determinants are multifactoral and thus too complex to demonstrate in this type of experiment.

In studies of the multiplication of Chikungunya in the various A. albopictus geographic strains, marked quantitative differences in the amount of virus present were observed, however. For example, female mosquitoes from the Hawaii strain consistently yielded 6 to 8 logs of virus one week after infection, while the Viet Nam and Malaysian strains yielded several logs less after the same incubation period. This suggests strain differences (probably genetic) in the ability to replicate and possibly to transmit the virus within the same mosquito species.

Serologic studies of arbovirus infection among human population of the South Pacific

Several thousand human sera were obtained from populations living on islands in the South Pacific and in Southeast Asia. Preliminary studies with these specimens and with convalescent sera of patients with Group A arbovirus infections indicated that the plaque neutralization method was considerably more specific for identifying Group A arbovirus infections than the hemagglutination-inhibition test which has been used extensively in the past in serologic surveys. Using the plaque neutralization method, specific antibodies to Chikungunya, Ross River and Sindbis viruses were identified. Results of these studies indicate a high prevalence of Ross River neutralizing antibodies among population groups living in West Irian,

New Guinea and several of the Solomon Islands. Ross River virus is the presumed etiologic agent of epidemic polyarthritis and has not been reported occurring outside of Australia. Evidence of Chikungunya infection was found in human populations living in Malaysia, Borneo, Java, Celebes, and the Philippines. Antibody rates to Sindbis virus were generally low, and no evidence of human infection with Getah or Bebaru viruses was found. Work is now in progress to attempt to map the geographic distribution of these 5 agents in the South Pacific, based on the prevalence of human neutralizing antibodies.

Antigenic relationships among Phlebotomus fever group arboviruses

These studies were a continuation of those begun by the principal investigator at the Middle America Research Unit. At present, a total of 22 distinct viral agents from Africa, the Mediterranean region, Central Asia, and tropical America are included in the Phlebotomus fever arbovirus group. A number of these agents have never been described and a complete serologic study of the group has not been done. Cross-neutralization, complement fixation, and hemagglutination-inhibition tests were performed, using specific hyperimmune rodent sera prepared against each of the 22 agents, in collaboration with workers at the Middle America Research Unit and Yale Arbovirus Research Unit. A manuscript was prepared describing the antigenic relationships among members of the Phlebotomus fever goup and the implications of these data for the epidemiology of sandfly fever.

Multiplication of Phlebotomus fever group arboviruses in mosquitoes

Twenty-two Phlebotomus fever group viruses were inoculated intrathoracically into A. albopictus and C. fatigans. Multiplication in one or both mosquito species was demonstrated with 8 of the agents. Mean titers of positive mosquitoes inoculated with the 8 agents and maintained at 32°C for 10 days varied from $10^{2.5}$ to $10^{4.3}$ plaque forming units per insect. Interestingly, 2 of the viruses included in the Phlebotomus fever group have been isolated repeatedly from mosquitoes but never from sandflies, suggesting that the Phlebotomus fever serogroup is composed of mosquito as well as sandfly-borne agents.

Neutralization studies of sandfly fever convalescent sera

Convalescent sera from sandfly fever (Sicilian) human volunteers, obtained from Fort Detrick, were tested by the plaque neutralization method against 6 different Phlebotomus fever group viruses. Sera from these patients had substantial levels of specific neutralizing antibodies (1:20 to 1:1280) 28 and 56 days following infection, suggesting long-lasting immunity. In contrast, the same sera failed to neutralize 5 other Old World Phlebotomus fever group viruses, indicating that immunity to sandfly fever is probably type specific.

Studies of transovarial transmission of Bunyamwera supergroup viruses in mosquitoes

Recent reports of transovarial transmission of La Crosse virus (California encephalitis group) by Aedes triseriatus, prompted us to attempt vertical transmission of La Crosse with another Aedes species. Using experimentally infected (intrathoracic inoculation) A. albopictus females, we demonstrated that La Crosse virus is also transovarially transmitted in this mosquito species, although at a lower frequency than obtained with A. triseriatus.

In view of the La Crosse data as well as evidence that viruses of the Phlebotomus fever group are also vertically transmitted, it was decided to test the possibility of transovarial transmission with other Bunyamwera supergroup viruses. To date we have been unable to demonstrate transovarial transmission of 4 other Bunyamwera supergroup viruses (Cache Valley, Batai, Itaporanga and Arumowot) by experimentally infected C. fatigans and/or A. albopictus. These results suggest that there is something unique about La Crosse virus, permitting vertical transmission, in contrast to most other mosquito-borne viral agents.

Significance to Biomedical Research and the Program of the Institute

Chikungunya, a group A arbovirus, has been responsible for major epidemics of dengue-like illness among human populations in Africa, India, the Philippines, and several countries of Southeast Asia. Since dengue and Chikungunya infection are similar clinically and as both agents may occur simultaneously in the same area, differentiation of illness produced by the two viruses is of obvious public health importance. Like dengue, Aedes aegypti has been implicated as the principal urban vector during epidemics of Chikungunya disease. Despite a common vector, overlapping geographic distribution, and clinical similarity with dengue, however, isolations of Chikungunya virus from other mosquito genera (Anopheles, Culex and Mansonia), its sporadic appearance, as well as its occurrence in dengue-free areas of Africa suggest that the ecology of the two viruses is different. These studies are designed to learn more about the epidemiology of Chikungunya disease.

Ross River, a second Group A arbovirus, is the presumed etiologic agent of epidemic polyarthritis, a disease of some public health importance in Australia. Results of our serologic surveys indicate that the virus also occurs in New Guinea, West Irian and several of the Solomon Islands, extending its known geographic distribution. Studies of epidemic polyarthritis patients in Australia suggest that the disease may represent a hypersensitivity reaction following Ross River virus infection, since persons manifesting arthritic symptoms already have antibodies to the agent and attempts at virus isolation have been unsuccessful. In this respect, epidemic polyarthritis is analogous to the arthritis associated with rubella infection.

La Crosse virus, a member of the California encephalitis arbovirus group, has been the agent most frequently associated with human cases of California encephalitis in the United States. Recent evidence of transovarial transmission of this virus by Aedes triseriatus in Wisconsin suggests an efficient mechanism for overwintering of the agent and maintenance in the mosquito population. This is the first evidence of transovarial transmission of an arbovirus by mosquitoes. The possible genetic implications of this mechanism are intriguing, for if vertical transmission is the sole maintenance mechanism for the virus, then transmission rates must approach 100% in order to insure virus survival. This might suggest a chronically infected subpopulation, transmitting the virus to their offspring and future generations, within the total mosquito population, thus maintaining the virus at a low level of endemicity. Experiments to test this possibility, using A. albopictus as a model, are in progress.

Sandfly fever has long been recognized as a disease of considerable public health and military importance in the Middle East and Central Asia. Recent studies indicate that Phlebotomus fever group viruses and the disease also occur in Africa and tropical America. Previous studies of sandfly fever have been limited mainly to clinical observations of patients, because of difficulties in culturing the virus and lack of a specific serologic test for determining immunity. Now, however, with the availability of tissue culture for primary virus isolation and for use in plaque neutralization tests, the necessary laboratory tools are available for a thorough epidemiologic study of sandfly fever.

Proposed Course of the Project

Serologic surveys by plaque neutralization test will be completed on human sera from various Pacific islands and Southeast Asia to map the distribution of Group A arbovirus activity in the area. It is hoped that this method will also allow us to map the distribution of certain Group B viruses, such as Japanese and Murray Valley encephalitis. The techniques developed in these studies now give us the capability to carry out field epidemiologic investigations in the event of an epidemic caused by one of these virus agents in the South Pacific.

Laboratory studies on the genetics of susceptibility to oral Chikungunya infection in A. albopictus will be continued. The apparent differences in ability to replicate the virus among different strains of this mosquito species is significant, since it may indicate differences in vector potential. Such differences if they also occur with other viruses and other mosquito species might offer a means of genetic manipulation and biologic control.

Collaborative studies are planned with workers at the Institute of Public Health Research, School of Public Health, University of Teheran, Iran to test sera from human populations living in areas of endemic sandfly fever. If these studies are successful and an endemic area of the disease is identified, further epidemiologic investigations are planned.

Work on the genetics of La Crosse transovarial transmission with laboratory colonies of A. albopictus will be continued.

Honors and Awards

None

Publications:

Tesh, R.B. and Johnson, K.M.: Vesicular stomatitis. In Hubbert, W.T., McCulloch, W.F. and Schnurrenberger, R.P. (eds): Diseases Transmitted from Animals to Man. Sixth Edition. Charles C. Thomas, Springfield, Illinois, 1974.

Tesh, R.B., Ackerman, L.J., Dietz, W.H. and Williams, J.A.: Angiostrongylus costaricensis in Panama. Prevalence and pathologic findings in wild rodents infected with the parasite. Am. J. Trop. Med. Hyg. 22:348-356, 1973.

Martin, D.H., Chaniotis, B.N., and Tesh, R.B.: Host preferences of Deinocerites pseudes Dyar & Knab. J. Med. Entomol., 10:206-208, 1973.

Tesh, R.B., Chaniotis, B.N., Peralta, P.H., and Johnson, K.M.: Ecology of viruses isolated from Panamian phlebotomine sandflies. Am. J. Trop. Med. Hyg., 23:258-269, 1974.

Noble, G.A. and Tesh, R.B.: Monoecocestus diplomys sp. n. (Cestoda: Anoplocephalidae) from the rat Diplomys darlingi. J. Parasitol. 1974 (in press).

Tesh, R.B., Peralta, P.H., Shope, R.E., Chaniotis, B.N., and Johnson, K.M.: Antigenic relationships among Phlebotomus fever group arboviruses and their implications for the epidemiology of sandfly fever. Am. J. Trop. Med. Hyg. 1974 (in press)

Chaniotis, B.N., Correa, M.A., Tesh, R.B., and Johnson, K.M.: Horizontal and vertical movements of phlebotomine sandflies in a Panamanian rainforest. J. Med. Entomol. 1974 (in press)

Serial No. NIAID 67D

1. Laboratory of Parasitic Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on the natural history of Toxoplasma gondii, the mechanism of its transmission to man, and its public health importance in the Pacific area.

Previous Serial Number: Same

Principal Investigator: Dr. Gordon D. Wallace

Other Investigators: Dr. Alistair Philip, Department of Pediatrics, University of Hawaii School of Medicine, Honolulu, Hawaii

Cooperating Units: Dr. John H. Cross, U.S. Naval Medical Research Unit No. 2, Taipei, Taiwan.

Man Years: NIAID staff

Total:	5/12
Professional:	5/12
Other:	0/12

Project Description:

Objectives

To determine the method, or methods, by which man becomes infected with T. gondii.

To identify subclinical cases of congenital infection for long term observation on the development of sequelae.

Methods Employed

Seroepidemiology. Reports in the literature indicate that Chinese living in certain areas of Southeast Asia have a remarkably lower incidence of toxoplasmosis than other ethnic groups in the same area. To provide more information on this, sera for epidemiologic studies were obtained from selected groups of ethnic Chinese and aborigines in Taiwan, Chinese and Indonesians in Indonesia, and Chinese and Tahitians in Tahiti.

Congenital toxoplasmosis. Information from Toxoplasma dye tests on sera collected from the umbilical cords of about 1000 babies born on Oahu, indicated that Filipino babies were at a much higher risk of becoming congenitally infected with Toxoplasma than other ethnic groups in Hawaii. Also, the majority of documented cases of congenital toxoplasmosis in Hawaii were in Filipino babies, although filipinos are a minority group. Consequently a prospective study of congenital toxoplasmosis in Filipino babies born on Oahu was undertaken. Cord blood was collected from most of the Filipino babies born on Oahu from Feb. to Nov. 1973. Sera were tested for Toxoplasma antibody by the dye test and attempts were made to collect an additional blood specimen four to six months after birth from each baby that had a cord-blood titer of 1:128 or greater. A careful clinical evaluation including psychophysiological testing was planned for all babies with serologic evidence of infection.

Major Findings

Seroepidemiology. Of one group of 132 primitive aborigine natives of various ages in Taiwan, Toxoplasma antibody ($\geq$1:16) was found in only 4. Of another group of sera from 47 hospitalized patients (mostly adults) in Taiwan, only two were positive. Of 23 Tahitians and 21 Chinese living in Tahiti that were tested, Toxoplasma antibody was detected in 70% and 86% respectively. In Indonesia, 50% of 38 Indonesian medical students were found to have Toxoplasma antibody, whereas antibody was detected in only 26% of 86 Chines medical students tested at the same university at the same time. The reasons for the geographic variation in the findings in the Chinese tested were not determined.

Congenital toxoplasmosis. Sera from umbilical cords of 522 Filipino babies were tested at a 1:128 dilution. Of these, 70 were positive. The distribution of titers was as follows: 128-256, 30; 512-1024, 20; 2048-4096, 13; 8000-16,000, 6; 32,000,1. Follow-up specimens from 29 babies have been tested and all but one had a significant drop in titer. One had a titer increase from 1:512 to 1:8192. This baby has not yet been examined.

Significance to Biomedical Research and the Program of the Institute

Although considerable progress has been made during the past four years in understanding the natural history and transmission of Toxoplasma, some of the specific factors responsible for human infection have not been determined. It would be of considerable interest to determine the reasons for the relatively low prevalence of human infection in certain ethnic groups, such as the aborigines living under primitive conditions in Taiwan.

Published data indicate that 60% of babies congenitally infected with Toxoplasma will be asymptomatic at birth. It can be predicated that chorioretinitis and other sequelae will become evident in some of these babies during the first 6 months of life. However data are needed on the possibility of eventual development of sequela in infected babies that appear

to be normal for a year or longer. Information from one prospective study suggests that 100% of congenitally infected babies will eventually manifest intellectual impairment.

Proposed Course of the Project

Most of the work on Toxoplasma will be terminated in the near future. However new studies might be initiated if unique opportunities to obtain useful information become evident.

Honors and Awards:

Dr. Wallace served as Associate Clinical Professor of Tropical Medicine and Medical Microbiology in the University of Hawaii School of Medicine, and assisted clinical laboratory directors and physicians in Hawaii with diagnostic problems related to toxoplasmosis.

Publications:

Wallace, G.D.: Intermediate and transport hosts in the natural history of Toxoplasma gondii. Am. J. Trop. Med. Hyg., 22:456-464, 1973.

Wallace, G.D., Zigas, V., and Gajdusek, D.C.: Toxoplasmosis and cats in New Guinea. Am. J. Trop. Med. Hyg., 23:8-14, 1974.

Serial No. NIAID 67H

1. Laboratory of Parasitic Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on dengue

Previous Serial Number: Same

Principal Investigator: Dr. Leon Rosen

Other Investigators: Dr. Duane Gubler, Dr. Lloyd Rozeboom, Dr. Winton Barnes, Dr. P.F.D. Van Peenen, Dr. Teong Wah Lim

Cooperating Units: School of Hygiene and Public Health, Johns Hopkins University, Baltimore; Government of Niue, Niue Island; U.S. Naval Medical Research Unit No. 2, Jakarta, Indonesia; Government of the Kingdom of Tonga, Nukuolofa, Tonga; Institute for Medical Research, Kuala Lumpur, Malaysia.

Man Years:	NIAID Staff	Guest Worker
Total:	24/12	6/12
Professional:	24/12	6/12
Other:	0/12	0/12

Project Description

Objectives

1. To study the epidemiology of dengue and dengue hemorrhagic fever in the simple ecologic environment of Pacific Islands.

2. To study the relative susceptibility of various species of mosquitoes to infection with dengue viruses.

3. To improve laboratory methods for the detection of dengue viruses and their antibodies.

Methods Employed

Epidemics of dengue are studied as they occur on Pacific islands. During the year, investigations were carried out on an outbreak of dengue which occurred in Tonga.

Also, various species of mosquitoes and Macaca monkeys were infected experimentally with strains of dengue virus (including the four prototypes) which had not been potentially modified by previous passage in mice or cell cultures.

Various virologic techniques previously developed for use with non-arthropod borne viruses were used in an attempt to improve the techniques currently available for work with the dengue viruses.

Major Findings

Tonga

An outbreak of dengue was detected on the island of Tongatabu, Tonga in March, 1974 and field investigations were undertaken with two principal objectives in mind, 1) to study variations in viremia levels from person to person and in the same person on different days of illness, and 2) to evaluate the role of the local Aedes species of the scutellaris complex in the transmission of the disease.

The investigations were just beginning as this report was being written, but it is presumed that the outbreak is due to dengue 2 virus, the only dengue type isolated from Pacific islands during the last 3 years.

Niue

Further study of sera collected from Niue Island indicated that children who survived severe dengue there had significantly higher homologous dengue neutralizing antibody titers than did children of comparable age who had ordinary dengue. Also, persons who resided in households in which severe or fatal cases had occurred were more likely to have high antibody titers than were persons from other households. These findings suggest that severe dengue may be the result of unusually high viremia levels which in turn may reflect either increased virus virulence or, alternatively, increased host susceptibility.

Panama

Previous serologic studies by the principal investigator indicated that dengue type 2 had occurred in Panama during World War II. At the time these studies were carried out only dengue types 1 and 2 were known. Some hemagglutination-inhibition (HI) positive sera from Panama were negative for neutralizing antibody against these dengue types but it was hypothesized that

this finding was a result of the relative insensitivity of the dengue 2 neutralization test (which was carried out in mice) as compared to the HI test. Recently, the sera from Panama were re-examined for plaque reduction neutralizing antibody against all 4 dengue types and it was found that the HI positive sera which previously had no dengue 2 neutralizing antibody as measured in the mouse test, also had no dengue 2 antibody in the plaque reduction neutralization test, but they did have dengue 3 antibody. Further serologic studies indicated clearly that the disease which occurred in Panama during World War II was due to both dengue 2 and dengue 3. This documents the presence of the latter type in the New World some 20 years prior to its previous first known occurrence.

Isolation of dengue viruses from different geographic areas and from clinical diseases of varying severity

As a prelude to planned study of the virulence of dengue viruses as they exist in nature, a collection has been started of dengue strains from different geographic areas and from cases of different clinical severity. Only strains isolated directly from the original human serum in mosquitoes will be employed in the planned studies. During the past year dengue strains were isolated from human sera obtained from Indonesia, Malaysia, the Philippines, Puerto Rico, and Tahiti. All four serotypes were represented.

Comparative susceptibility of different mosquito species to dengue

In an attempt to find an explanation for the apparent virulence of dengue during the Niue Island outbreak, Aedes aegypti from the island and Aedes cooki, the local scutellaris species, were colonized and exposed to a South Pacific strain of dengue type 2 by ingestion. Surprisingly, the aegypti appeared unusually resistant to infection (as compared with aegypti from another source), whereas the cooki appeared unusually susceptible (as compared with other species of the scutellaris complex). This was the opposite result of what had been anticipated.

Improvement in dengue laboratory techniques

Extensive experiments were carried out to compare the fluorescent antibody technique for the detection of infected mosquitoes to their detection by assay in LLC-MK_2 cells. It was found that for dengue types 1 and 2 the two methods gave essentially comparable results. For dengue types 3 and 4 however, a significant number of mosquitoes were positive in LLC-MK_2 cells and negative by fluorescent antibody assay.

In order to improve the sensitivity of the mosquito inoculation technique, experiments were undertaken to determine if dengue virus in human sera could be concentrated by ultracentrifugation. It was found possible to concentrate virus in this way and the procedure was used to isolate virus from human serum specimens from the 1969 Tahiti type 3 outbreak which heretofore had failed to yield isolates by all previously known methods.

Significance to Biomedical Research and the Program of the Institute

Dengue viruses are increasing in significance as a cause of morbidity and mortality in Southeast Asia and Pacific islands - probably because of rapidly increasing urbanization and travel in that geographic area. Mosquito vectors of these viruses are ubiquitous in the same area, which includes many islands belonging to, or controlled by, the United States. Obviously, further understanding of the epidemiology of dengue and of the vectors of the disease would be of value.

Proposed Course of the Project

Further field studies on dengue outbreaks will be undertaken as appropriate opportunities occur. An attempt will also be made to write up for publication the extensive amount of data already on hand from outbreaks investigated previously.

Honors and Awards:

Dr. Rosen was appointed to the Ad Hoc Study Group on Viral and Rickettsial Diseases of the U.S. Army Medical Research and Development Command, was asked to serve as an advisor to the PAHO Scientific Advisory Committee on Dengue and was elected Vice President of the American Society of Tropical Medicine and Hygiene. He continued to serve as a member of the editorial board of Intervirology; as a member of the Viral Diseases Panel, U.S.-Japan Cooperative Medical Science Program; on the Executive Committee of the American Committee on Arthropod-borne Viruses; as Clinical Professor of Tropical Medicine and Medical Microbiology, University of Hawaii School of Medicine; and as a member of the Affiliate Graduate Faculty, University of Hawaii School of Public Health.

Publications:

Rozeboom, L.E., Rosen, L., and Ikeda, J.: Observations on oviposition of Aedes (S.) albopictus Skuse and A. (S.) polynesiensis Marks in nature. J. Med. Entomol. 10:397-399, 1973.

Char, D.F.B. and Rosen, L.: Eosinophilic meningitis. In Gellis, S.S. and Kagan, B.M. (eds.): Current Pediatric Therapy - 6. Philadelphia, W.B.Saunders Co., 1973, p. 554.

Char, D.F.B. and Rosen, L.: Angiostrongyliasis. In Gellis, S.S. and Kagan, B.M. (eds.): Current Pediatric Therapy - 6. Philadelphia, W.B. Saunders Co.

Saugrain, J., Moreau, J.P., and Rosen, L.: L'epidemie de dengue de Tahiti en 1971. Bull. Soc. Pathol. Exot. 66:381-385, 1973.

Rosen, L.: Reoviruses. In Hubbert, W.T., McCullogh, W.F. and Schnurrenberger, P.R. (eds.): Diseases Transmitted from Animals to Man, Sixth Edition, 1974, (in press)

Rosen, L.: Angiostrongyliasis. In Hubbert, W.T., McCulloch, W.F. and Schnurrenberger, P.R. (eds.): Diseases Transmitted from Animals to Man, Sixth Edition, 1974, (in press)

Barnes, W.J.S. and Rosen, L.: Fatal hemorrhagic disease and shock associated with primary dengue infection on a Pacific Island. Am. J. Trop. Med. Hyg. (in press)

Rosen, L.: Reoviruses. In Lennette, E.H., Spaulding, E.H. and Truant, J.P. (eds.): Manual of Clinical Microbiology - Second Edition (in press)

Rosen, L.: Reoviruses. In Standardized Methods for Veterinary Microbiology, to be published by the National Academy of Sciences (in press)

Serial No. NIAID 67 I

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Susceptibility and Resistance of Mosquitoes to Dengue

Previous Serial Number: Same

Principal Investigator: Dr. Duane J. Gubler

Other Investigators: Dr. Leon Rosen

Cooperating Units: Department of Tropical Medicine and Medical Microbiology, University of Hawaii School of Medicine, Honolulu.

Man Years:	NIAID Staff	Guest Worker
Total:		6/12
Professional:		6/12
Other:		0/12

Project Description:

Objectives:

1. To select strains of A. albopictus which are either highly resistant or highly susceptible to infection with dengue viruses.

2. To study the genetic basis of resistance to infection.

3. To study replication and distribution of dengue virus in resistant and susceptible mosquitoes.

Methods Employed

Attempts to infect Aedes (S.) albopictus and other species of Stegomyia orally with dengue viruses usually result in rates of infection of less than 100 percent. Strains of A. albopictus from different geographic areas will be screened for susceptibility to infection with dengue viruses. Those strains showing the highest natural resistance will be selected for resistance by feeding on suspensions with high virus concentration whereas selection pressure for increased susceptibility will be placed on those strains showing the highest natural susceptibility by feeding on suspensions with low virus concentration. Reciprocal and backcrossing experiments will be made to determine the type of inheritance controlling vectorial capacity after pure lines have been established.

Studies will be made on the replication and distribution of dengue virus in mosquitoes in relation to resistance and susceptibility. Methods employed for these studies will include virus titration of various mosquito organs and tissues by mosquito assay and determination of the site of virus replication by fluorescent antibody and, possibly, electron microscopy.

Major Findings

To date, 11 different geographical strains of Aedes albopictus have been screened for susceptibility to dengue viruses. Most of these strains showed comparable infection rates with dengue 2 after feeding on an artificial feeding suspension which had a titer of approximately 5×10^6 mosquito ID_{50} per ml. However, 2 strains (Israel and Philippine) consistently showed lower infection rates and 1 strain (Vietnam) has consistently showed a higher infection rate than the others. Selection of these and some of the other strains is now in the F_3 and F_4 without any evidence of increased susceptibility or resistance.

Experiments have been carried out to determine whether the resistance observed in some strains can be overcome by increasing the virus dose ingested with the blood meal. In several tests, 100% of the Oahu A. albopictus have been infected after feeding on a suspension containing over 10^8 MID_{50} per ml. However, this was still not enough to infect all of the Israel and Philippine mosquitoes. These results suggest that the resistance observed in the Israel and Philippine strains is real, but that it may be more realistic to think of resistance in terms of threshold levels of infections rather than in terms of genetically controlled absolute resistance.

Preliminary data suggested that age of the mosquito might influence infection by dengue virus. Experiments to test this influence have shown that there is no difference in susceptibility of mosquitoes of different ages.

Recent studies on the growth characteristics of dengue virus in Aedes albopictus have concentrated on following the development of infection in the salivary glands by the direct fluorescent antibody technique.

The first signs of infection were observed 6 to 8 days after feeding and consisted of small foci on 1 or 2 lateral lobes. Infected fat cells could also be observed adhering to the surface of the glands on these days. The infection nearly always began in the lateral lobes, but not in any particular portion of these lobes. Once infection had been established, it spread in all directions until the whole lobe was involved. The median lobes often remained negative for 20 days or more, but eventually became infected.

The percentage of infected salivary gland lobes increased with time until day 12 when over 80% showed some specific fluorescence. About 20% of these lobes were fully infected throughout their length, the rest having just a portion of the tissue infected. By day 20 to 26, all lobes were usually infected, although an occasional median lobe remained negative. Most of the lobes that were infected had no evident negative tissue at this time.

Virus was observed to aggregate in the salivary glands, beginning about day 18. There was no apparent pattern to this aggregation, but by day 26, clumps of antigen could be observed throughout the length of most infected lobes. There was a concomitant decrease in specific fluorescence in other parts of the lobe when this occurred, in many cases giving the impression of decreased virus content.

Significance to Biomedical Research and the Program of the Institute

Dengue viruses are widespread in Asia and the Pacific and outbreaks of the disease associated with severe hemorrhage or shock make dengue one of the most important public health problems of that area. Control of the vector is presently the only available means of controlling the disease. One possible method of control would be to replace a susceptible vector species with a refractory strain of the same species. Another possible approach would be to increase the resistance of a natural vector population by introducing the gene controlling this factor into the population. Theoretically, either of these methods, which depend upon selection of resistant mosquito strains in the laboratory, could interrupt transmission and bring about permanent disease control.

Proposed Course of the Project

Since no evidence has been obtained of genetic control of susceptibility of mosquitoes to dengue this aspect of the project will soon be terminated. Work will continue on the mechanism of salivary gland infection and its implications for the ability of mosquitoes to transmit infection.

Honors and Awards

Dr. Gubler was appointed as a short-term consultant to the World Health Organization to advise on a filariasis project in Indonesia.

Publications:

None

Serial No. NIAID 67J

1. Laboratory of Parasitic Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Observations on a recently discovered coccidian parasite that has similarities to Sarcocystis and Toxoplasma.

Previous Serial Number: Same

Principal Investigator: Dr. Gordon D. Wallace

Other Investigators: None

Cooperating Units: Dr. J. Frenkel, School of Medicine, University of Kansas, Dr. R. Hackman, School of Public Health, University of Washington.

Man Years: NIAID Staff

Total:	6/12
Professional:	6/12
Other:	0/12

Project Description:

Objectives

To elucidate the life cycle and biologic characteristics of the new parasite, particularly its relationship to Toxoplasma and its potential to infect man.

Methods Employed

The parasite, which will be referred to as WC1170, was isolated by inoculating oocysts morphologically similar to Toxoplasma, found in the feces of a naturally infected cat, into laboratory mice. Such mice developed chronic infections with the formation of Toxoplasma-like cysts in skeletal muscle. When chronically infected mice were fed to susceptible cats, the parasite reproduced in intestinal epithelium, resulting in fecal shedding of oocysts. The host range of WC1170 was studied by exposing rats, dogs, monkeys, pigeons and Japanese quail to the oocyst stage and observing them for serologic and histopathologic evidence of infection. Two other isolates from feces of stray cats appeared to be strains of the same parasite. These

strains, WC1170, and Toxoplasma isolated from cats were compared immunologically by cross immunity studies in cats and mice, and antigenically by an indirect fluorescent antibody test (IFAT) and the Toxoplasma dye test. Also, antigenic comparisons by two-dimensional immunoelectrophoresis were made by Dr. Hackman in Washington.

Major Findings

The three strains of the parasite appeared to be morphologically, biologically and antigenically identical, but they were distinctly different from Toxoplasma. There was good cross immunity among the strains in cats, but little or no immunity to Toxoplasma and vice versa. Toxoplasma-like cysts were plentiful in skeletal muscles of WC1170-infected mice, but absent or seldom observed in brains. It was not possible to transmit the parasite from chronically infected mice to susceptible mice by feeding or inoculating cysts. Mice and rats, but not cats, were easily infected with oocysts, thus indicating that a cat rodent cycle is obligatory for the propagation of the parasite.

There was serologic evidence of infection following exposure to oocysts in mice, rats, and dogs, but not in birds (quail and pigeons), or in Macaca irus monkeys. When muscles from exposed rats and birds were tested for infectivity by feeding to cats, rat muscles but not bird muscles were found to be infective (cat shed oocysts). Some mice became ill following inoculation with large numbers ($\geq 10^6$) of oocysts, but not the other animals tested. Myositis and myocarditis was extensive in some mice.

Sera from 32 people, all adults whose occupations required close contact with cats, and from 65 military dependents between 1 and 18 years of age, living on Oahu, Hawaii, were tested by IFAT for antibody to WC1170 zoites. Titers ranging between 1:16 and 1:64 were found in sera of 9 of the adults and in 7 of the children (ages 1-15 years). All but two sera (titers 1:16) that reacted with WC1170 antigen were also positive for Toxoplasma in the dye-test.

Early in the course of observations on WC1170, but not since, sarcocysts morphologically typical of those described for S. muris were found in skeletal muscles of a few mice inoculated with WC1170 oocysts. Similar sarcocysts were also found in mice inoculated with WC1170 oocysts in Dr. Frenkel's laboratory in Kansas City. However in spite of an extensive search for other parasites that might have been in the inoculum, but were overlooked, and exposure of various strains of mice and mice on cortisone acetate to WC1170 oocysts, the presence of the S. muris-like sarcocysts has not been explained.

In conclusion, the WC1170 parasite has biologic similarities to Sarcocystis and certain morphologic and antigenic similarities to Toxoplasma, yet it is uniquely different from both parasites. Its host range appears to be limited, it is relatively non-pathogenic and there is no evidence that it is of medical importance.

Significance to Biomedical Research and the Program of the Institute

Information on this parasite is of interest because of its similarities to both Toxoplasma and Sarcocystis, particularly since the life cycle of Sarcocystis, which is known to infect man, has been obscure.

Proposed Course of the Project

Because it now appears that the parasite is of little or no medical importance, I will terminate my research project in the near future. However I will continue to collaborate with Drs. Frenkel and Hackman. Dr. Frenkel is interested in the biology of the parasite and Dr. Hackman is interested in exploring its antigenic relationship to Toxoplasma and to determine if it has an antigenic relationship to Sarcocystis species.

Honors and Awards:

See NIAID 67D

Publications:

Wallace, G.D.: Sarcocystis in mice inoculated with Toxoplasma-like oocysts from cat feces. Science, 180:1375-1377, 1973.

Serial No. NIAID 67K

1. Laboratory of Parasitic Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on Angiostrongylus: Experiments on the host range of A. costaricensis, and the evaluation of a fluorescent antibody test for the diagnosis of angiostrongyliasis

Previous Serial Number: Same

Principal Investigator: Dr. Gordon D. Wallace

Other Investigators: Dr. Leon Rosen

Man Years:

	NIAID Staff
Total:	1/12
Professional:	1/12
Other:	0/12

Project Description:

Objectives

To determine if Pacific island rodents and mollusks can serve as suitable hosts for A. costaricensis, a rodent metastrongylid of medical importance in Central America.

To develop a serologic test for the diagnosis of human disease caused by Angiostrongylus.

Methods Employed

Infective third-stage larvae of A. costaricensis were obtained from Costa Rica and the parasite was maintained in the laboratory in the cotton rat, Sigmodon hispidus and in an aquatic snail, Biomphalaria glabrata. Laboratory raised Rattus rattus, R. norvegicus, R. exulans, S. hispidus and Mus musculus were exposed to various doses of third-stage A. costaricensis larvae and observed for fecal shedding of first-stage larvae. Three species of terrestrial mollusks, two of which are common on Pacific islands, were exposed to first-stage larvae of A. costaricensis and examined for third-stage larvae 5 to 9 weeks later. As controls, some of each species of mollusk were exposed to A. cantonensis.

Laboratory rats and mice infected with third-stage larvae of A. cantonensis or A. costaricensis were bled periodically for antibody determinations by an indirect fluorescent antibody test (IFAT) in which frozen sections of adult worms were used as antigen. Sera from human cases of eosinophilic meningitis (proven by spinal tap) in Tahiti and from "normal" people elsewhere, probably not exposed to A. cantonensis, were also tested.

Major Findings

Of the three species of Pacific-island rats tested (R. rattus, R. norvegicus, R. exulans), only R. rattus shed larvae in quantities and for periods of time sufficient to have the potential of being an important definitive host of A. costaricensis. Two of five mice exposed to A. costaricensis survived the prepatent period and shed larvae, one in relatively large numbers. However both mice succumbed to the parasite one to three weeks later. Experiments in which rats were exposed to A. cantonensis following a patent infection with A. costaricensis, and vice versa, revealed little or no cross immunity between the parasites. First-stage A. costaricensis larvae developed to infective third-stage (proven by inoculation into rats) in all of the species of mollusks tested, but in much smaller proportions than did A. cantonensis.

In an effort to determine if A. costaricensis occurs in the U.S., 142 wild cotton rats trapped in South Texas were examined for natural infection with this parasite. All were negative.

IFA tests on sera from 10 mice experimentally infected with A. cantonensis demonstrated the appearance of antibody in all mice between 10 and 22 days after exposure. Peak titers of 1:512 to 1:1024 were reached within 3 to 6 weeks and generally diminished to between 1:64 and 1:256 by 4 months, the last time period tested. All but 1 of 17 sera collected within a few days to several months after onset of illness from confirmed human cases of eosinophilic meningitis in Tahiti had titers of 1:16 to 1:512. In 11 cases from which specific information on date of onset of illness was available, it was determined that the low-titered sera were collected either 7 to 10 days or three to eight months after onset. However, all but 1 of 24 sera collected from Samoans, presumed to have never been exposed to A. cantonensis, reacted to the antigen in dilutions ranging between 1:16 and 1:64. Also, sera from one of three laboratory personnel, also presumed not to have had exposure to the parasite, reacted to the antigen at a dilution of 1:128. Use of specific immunoglobulin conjugates (IgG or IgM), heat inactivation of serum, or use of sections of third-stage larvae as antigen, did not appear to improve specificity.

Significance to Biomedical Research and the Program of the Institute

Since A. costaricensis was recently recognized as an important parasite of man in Central America, it was of interest to learn if species of rats and mollusks common to the Pacific area could serve as suitable hosts of the

parasite. A. cantonensis is widespread in the Pacific area and it is conceivable that A. costaricensis could be introduced or, in fact, already be present but not yet recognized.

Most cases of eosinophilic meningitis occurring in the Pacific area are presumed to be caused by A. cantonensis. However a specific diagnostic test for infection with A. cantonensis has not been developed. Evaluation of a fluorescent antibody test for this purpose is therefore indicated. The test might also have application in diagnosing human intestinal disease caused by A. costaricensis.

Proposed Course of the Project

Further observations will be made on the development and persistence of antibody to both A.cantonensis and A. costaricensis in experimentally infected animals, including observations on possible antigenic relationships between the parasites. Attempts are being made to obtain serial bleedings from patients with eosinophilic meningitis, so that antibody levels in relation to onset of illness can be determined. Also, sera from additional "normal" subjects will be tested.

Honors and Awards:

See NIAID 67D

Publications: None

LABORATORY OF IMMUNOLOGY

Summary 1

Serial No.

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PHS-NIH
Summary Statement
Office of the Chief
Laboratory of Immunology
July, 1, 1973 through June 30, 1974

During the past year the Laboratory of Immunology has continued the record of scientific achievement which has been established over the last several years. This was accompanied by a high degree of research productivity as evidenced by the publications of Laboratory members. The Laboratory has continued its role in the training of young scientists. This training function is associated with a substantial change in the temporary scientific staff of the Laboraotry. In addition to this turnover of younger scientists, which is detailed below, one of the senior members of the Laboraotry, Dr. E. Bruce Merchant, left to accept a position in the Bureau of Biologics, FDA. Three research associates, Dr. John Breitner, Dr. Benjamin Cohen and Dr. Michael Harrison completed their assigments at the beginning of this fiscal year. Four Visiting Fellows, Dr Mariella Carta, Dr. Raphael Sharon, Dr. Ramesh Srivastava and Dr. John van Boxel, one NIAID Special Fellow, Dr. Michael Gelfand, and one Guest Worker, Dr. Salvatore Carta, completed their training in the Laboratory during the past year. Many of these individuals made outstanding research contributions during their tenure in the Laboratory.

Several scientists and physicians joined the Laboratory this year. They are: Dr. Philip Cohen, Dr. Andrew Drexler and Dr. Fred Finkelman as Research Associates: Dr. Aftab Ansari and Dr. Ian Zitron as Visiting Fellows and Dr. Guido Forni and Dr. Hartmut Rühl as Guest Workers.

The members of the Laboratory play increasingly important roles in the immunologic community. Thus, Dr. Paul serves on the editorial boards of the Journal of Experimental Medicine, the Journal of Immunology, Immunochemistry, Immunogenetics, Immunological Communications, and the Scandanavian Journal of Immunology. He is also a member of the Board of Reviewers of Federation Proceedings. Dr. Mage is a member of the editorial boards of Federation Proceedings and Immunochemistry. Dr. Green and Dr. Inman are members of the board of reviewers of Federation Proceedings.

Dr. Mage is a member of the Allergy and Immunology Study Section of the Division of Research Grants, NIH, Dr. Green is a member of the Program Committee of the American Association of Immunologists and Dr. Paul served as chairman of the Nominating Committee of the American Association of Immunologists and is a member of the International Fellowship Review Panel of the Fogarty International Center.

Members of the Laboratory have given seminars, chaired symposia and been invited speakers at important meetings in the United States and Europe. Among these are the following:

Dr. Paul chaired symposia at the annual meeting of the Reticuloendothelial Society and at the Federation Conference on the Biology of Development and Aging. In addition, Dr. Inman , Dr. Shevach and

Dr. Paul chaired sessions at the annual FASEB meeting in Atlantic City. Dr. Paul presented major lectures at the Symposium on Cellular Selection and Regulation in the Immune Response at Woods Hole, Mass., the Symposium on Basic Immunology and Cancer at Cornell University Medical College, the Mid-West Immunology Conference in Chicago, the annual meeting of the Reticuloendothelial Society in Williamsburg, Virginia, the UCLA-ICN Conference on Immunology at Squaw Valley, California, the New York Academy of Sciences International Conference on Thymus Factors in Immunity and the Brook Lodge Conference on Tolerance: Mechanisms and Potential Therapeutic Applications.

Dr. Mage presented major talks at the Symposium on Cellular Selection and Regulation in the Immune Response at Woods Hole, and at the Federation Conference on Development and Aging. She chaired a workshop at the UCLA -ICN Conference on Immunology.

Dr. Green presented major lectures at the annual meeting of the Reticuloendothelial Society in Williamsburg, Va., the Symposium on the Sezary Cell at the Mayo Clinic and at the meeting of the International Academy of Pathology in San Francisco.

Dr. Shevach was an invited lecturer at the Mid-Winter Immunology Conference at Asilomar, California and at the Symposium on Cancer and Transplantation at Buffalo, N. Y. Dr. Mosier presented a major lecture at the Federation Conference on Development and Aging and Dr. Inman gave an invited presentation at the UCLA-ICN Conference on Immunology.

The Laboratory of Immunology has as its basic goal the exloration of fundamental mechanisms in cellular immunology, immunogenetics, immunochemistry, immunopathology and tumor immunology. To these ends, studies of the ontogeny and heterogeneity of lymphocytes, of the nature and function of antigen-binding membrane receptors of lymphocytes, and of the mechanisms of tolerance induction and lymphocyte interactions are being actively pursued. Major efforts are being exerted to elucidate the nature, genetics and mode of function of specific immune response gene products. Mechanisms of cell mediated cytotoxicity, of the nature of the genetic and chemical control of immunoglobulin synthesis and variability and of the activation of immunocompetent cells are being intensely studied.

ONTOGENY OF BONE-MARROW DERIVED LYMPHOCYTES

The origin and development of the class of mammalian lymphocytes which include precursors of antibody secreting cells (B lymphocytes) is still poorly understood, in part because no exclusive source organ for these cells has yet been identified. In recent years, several cell surface markers have been identified on these cells. Some of these markers (e.g. surface immunoglobulin) have clear physiologic roles, others have proposed roles (e.g. the receptor for the third component of complement (C)) and still others are completely without known function. Work from the Laboratory of Immunology had previously demonstrated that in the mouse the development of B lymphocytes bearing surface immunoglobulin

occurred well before lymphocytes bearing C receptors appeared. During the past year, it has been demonstrated that the duration of the lag in appearance of C receptor lymphocytes (CRL) is genetically controlled. AKR mice, in which the interval between the appearance of the two cell types is short, appeared to differ from DBA/2 mice, in which the interval is long, by two genes important in regulating the differentiation of CRL. Furthermore, it was demonstrated that one of these two genes was linked to the major histocompatibility (H-2) complex of the mouse. Mapping studies indicated that this gene (CRL-1) was either in the telomeric portion of the H-2 complex or between H-2 and the genes coding for the TL antigens. This is the first demonstration of a gene regulating B lymphocyte differentiation which is linked to the major histocompatibility complex. Delineation of this gene may have important implications for the analysis of the critical process of differentiation and acquisition of immunocompetence by B lymphocytes (Gelfand and Paul, LI/NIAID, Frank, LCI, NIAID and Sachs, IB/NCI.

IN VITRO ANTIBODY RESPONSE TO A "THYMUS-INDEPENDENT" ANTIGEN

The use of Ficoll, a synthetic polymer of sucrose and epichlorhydrin, as an immunogenic carrier for hapten derivatives has recently been proposed by McMaster. Studies from the Laboratories of Immunology and Microbial Immunity have shown that ε-2,4-dinitrophenyl-lysyl-Ficoll (DNP-Ficoll) is a powerful antigen in mice, that the response to this antigen is independent of thymus-derived lymphocytes and that the antibody response involves cells producing IgM and IgG antibody, respectively. In this respect DNP-Ficoll is unusual in that the response to most T-independent antigens involves little or no antibody of the IgG class.

Recently, it has been demonstrated that DNP-Ficoll is a powerful in vitro stimulant of a DNP-specific antibody response. This response is independent of thymus-derived (T) lymphocytes. Moreover, depletion of adherent cells from spleen cell populations does not diminish the response to DNP-Ficoll and often leads to an increased response. Thus, DNP-Ficoll is a T-independent antigen which appears to directly activate precursors of antibody secreting cells when it binds to their receptors, at least in certain critical concentration ranges. Moreover, since DNP-Ficoll is not a general B cell stimulant, the most logical explanation for its stimulatory characteristics is its resistance to degradation and the two dimensional array in which it presents antigenic determinants. Experiments are now in progress comparing the immunogenicity of Ficolls of varying sizes, degrees of substitution and degrees of degradation (or polymerization). Through such studies it is hoped that insight will be gained into the critical requirements for activation of precursors of antibody secreting cells. (Mosier, Johnson and Paul LI/NIAID and McMaster LMI/NIAID)

DISTINCT T AND B MITOGENS ISOLATED FROM POKEWEED MITOGEN

Lymphocytes from many species may be activated to DNA synthesis by a series of agents including the plant mitogens. The bulk of the mitogenic lectins activate thymus derived (T) lymphocytes preferentially, although

if aggregated onto solid matrices these agents sometimes acquire the capacity to stimulate thymus-independent (B) lymphocytes. One particularly important stimulant is pokeweed mitogen, which has been reported to activate both T and B lymphocytes. It has previously been demonstrated that 5 mitogenic lectins can, in fact, be extracted from pokeweed (Phytolacca americana). These lectins have been termed Pa 1-5. It has been shown that Pa 2-5, which have many common structural features, are stimulatory for T lymphocytes exclusively and that the B lymphocyte stimulatory capacity of crude pokeweed mitogen is due to its content of Pa-1, which is mitogenic for both B and T lymphocytes. Moreover, only Pa-1 causes immunoglobulin synthesis in vitro further supporting the contention that Pa-1 is the only B lymphocyte mitogen in pokeweed.

Studies are now in progress to delineate the chemical characteristics which confer on Pa-1 its B cell stimulatory capacity. In particular, large aggregates of Pa 2-5 are being prepared to determine whether presence of multiple lectin binding sites on a single particle is required for the stimulation of B lymphocytes. This is suggested not only by work on other aggregated lectins, as noted above, but also by the finding that Pa-1 aggregates spontaneously whereas Pa-2-5 exist as single polypeptide chains at neutral pH. Thus, the pokeweed family of mitogens appears to provide a particularly powerful tool to delineate the relative molecular requirements for T and B lymphocyte activation. (Waxdal and Basham, LI/NIAID).

COMMON CHARACTERISTICS OF A FAMILY OF PROTEINS, IMMUNE RESPONSES TO WHICH ARE CONTROLLED BY A SINGLY IMMUNE RESPONSE GENE.

The capacity to mount specific immune responses to several antigens is controlled by a series of immune response (Ir) genes which are linked to the major histocompatibility locus of the species. In the mouse, these genes occur in a region known as I, within the H-2 complex. Studies from the Laboratory of Immunology produced the first evidence that distinct Ir genes exist within the I region and that these distinct Ir genes control responsiveness to independent antigens. These experiments demonstrated that antibody production in response to immunization with a BALB/c IgA myeloma protein depended on an Ir gene distinct from the genes controlling responses to an IgG (γ_{2a}) myeloma protein. One of the striking findings in these experiments was that the antibody response which was observed was largely specific for idiotypic determinants on the myeloma protein.

Evidence has now been obtained that the Ir gene controlling responses to one IgG-κ myeloma protein probably controls responses to a total of 5 proteins of the same class. Thus, the product of Ir-IgG gene appears to identify antigenic determinants common to all IgG-κ proteins of the strain. This would indicate that the gene product recognizes allotypic determinants despite the fact that the predominant antibody response is specific for idiotypic determinants. This suggests that genetic control in this system operates not at the level of control of antibody specificity but, more likely, in the recognition of immunogenicity. In turn, this

suggests that the Ir gene involved in recognizing IgG myeloma proteins is principally expressed in T lymphocytes. (Lieberman and Paul, LI/NIAID and Potter, LCB/NCI).

DELINEATION OF MAJOR HISTOCOMPATIBILITY ANTIGENS OF GUINEA PIGS

The major histocompatibility (H) complex of guinea pigs has, until recently, been poorly understood. Recent studies from the Laboratory of Immunology demonstrating linkage between the H complex and the genes controlling specific immune responses have indicated the biologic importance of the H complex. Furthermore, the demonstration by LI scientists that alloantisera with primary specificity for the 2/13 class of guinea pig histocompatibility antigens can specifically inhibit recognition of antigens, responsiveness to which is controlled by specific Ir genes, emphasizes the need to delineate the guinea pig H system. This is particularly so as no comparable experimental blocking system yet exists in other species.

It has now been shown that strain 2 and strain 13 guinea pigs differ from each other at a locus tentatively termed the 2/13 locus but that they share the same specificity at another, probably linked, locus, the B, C, D locus. The B, C, D series of antigens has been shown to include the 3 antigens B, C and D and, very probably, one or two additional antigens which can be demonstrated in outbred guinea pigs. These antigens appear to bear an allelic relationship to each other, but there also exist one or two additional antigens tentatively identified by the number of the sera used in defining them (e.g. 336 and 340) which may define yet another locus or may represent public specificities. A series of breeding studies are now in progress to determine the genetic linkage existing between these loci and to help to define the relationship between the guinea pig H complex and the relatively well understood human and mouse major histocompatibilty complexes. (Shevach, LI/NIAID, Geczy and deWeck, Inselspital, Bern, Switzerland).

STRUCTURE OF GUINEA PIG HISTOCOMPATIBILITY ANTIGENS

Studies from the Laboratory of Immunology conducted over the last several years have demonstrated an important genetic and functional relationship between antigen-recognition by thymus-derived lymphocytes, immune response gene products and histocompatibility antigens. More precisely, it had previously been demonstrated that specific Ir gene product function in antigen recognition by guinea pig T lymphocytes can be blocked, *in vitro*, by antisera directed at histocompatibility antigens coded for at the 2/13 locus. Preliminary studies suggest that antisera directed at histocompatibility antigens coded for at the B locus do not block such antigen recognition. Thus, it is possible that the 2/13 class of antigens are linked to specific antigen recognition sites which might function as receptors on T lymphocytes. Consequently, attempts to chemically characterize these histocompatibility antigens were begun. Using enzyme catalyzed iodination of cell surface proteins, it has been

shown that the 2 and 13 antigens extracted from lymph node cells and, in the case of the 2 antigen, from a leukemia cell, have a molecular weight of approximately 25,000 daltons. On the other hand, the B antigen consists of 2 chains, one of which has a molecular weight of 45,000 daltons and the other a molecular weight of approximately 12,000 daltons. The 2/13 antigens have thus far been demonstrated only on lymphoid cells whereas the B antigen is much more widely distributed. Surface immunofluorescence studies reveal that 2 and B migrate independently in the plane of the membrane, indicating that they are independent molecules. Further chemical characterization of the 2 and 13 antigens are now in progress with the aim of establishing whether the molecules bearing these antigens also bear specific antigen-binding receptors. (Finkelman, Shevach, Green and Paul, LI/NIAID).

DEVELOPMENT OF A THEORETICAL MODEL TO EXPLAIN HETEROGENEITY OF ANTISERA WITH LIMITED NUMBERS OF V REGION GENES

One of the principal characteristics of the immune response to simple antigens is the high degree of molecular heterogeneity among the specific antibodies which are produced. This has led to the concept that the library of genes required to encode this variability must be enormous. Inman has recently developed a sophisticated mathematical model, based on multiple specificity of antibody molecules, which can explain the great heterogeneity and precise specificity of antisera produced in response to a given antigen and yet requires relatively small numbers of genes for the variable regions of heavy (H) and light (L) chains. Computer models have been devised to more fully determine the predictive value of this theory and experiments aimed at testing certain aspects of this proposal are now in progress. (Inman LI/NIAID)

CHARACTERIZATION OF PEPTIDES BEARING a LOCUS ALLOTYPIC DETERMINANTS

The variable region of the heavy (H) polypeptide chain makes the principal contribution to the specific antigen-binding site of antibodies. In the rabbit, allotypic forms of H chains exist which are controlled by alleles of the genes coding for the variable region of the H chain (V_H genes). The demonstration of stable genetic markers in the V_H region, transmitted according to Mendelian rules, has important implications for the genetic basis of antibody diversity. LI scientists previously isolated from antibodies of limited heterogeneity of a3 allotype, a peptide with a3 antigenic activity. These studies have now been extended to immunoglobulin of the a1 and a2 allotypes. In addition, chemical analysis of the peptide with a3 antigenic activity indicates a molecular weight of $\sim$ 6,000 daltons. This peptide loses antigenic activity and its molecular weight diminishes upon reduction, suggesting that antigenic activity is contributed to by two peptides linked by a disulfide bond. These studies should allow a precise localization of the portion of the variable region responsible for the allotypic antigenic activity and a delineation of the relation of these determinants to those portions of the variable region which form the specific binding site of antibody. In turn, this will be of importance in the determination of genetic control of antibody diversity and of the mechanism by which binding sites of antibodies are formed. (Mage, Carta, Young-Cooper, LI/NIAID).

IDENTIFICATION OF THE ORIGIN OF MALIGNANT HUMAN RETICULAR CELLS

There are a wide variety of tumors of the human lymphoid system. One principal problem in classifying these tumors has been a lack of understanding of the type of lymphoid cell which gives rise to the malignant cells in the various lymphoid tumors. Recently, techniques for identifying cell surface markers of human thymus-dependent (T) and thymus-independent (B) lymphocytes and of monocytes have been developed and applied to the determination of cell types in tissue sections. Much of the pioneering work in this field has been carried out by the Laboratory of Immunology sceintists. Through the use of these procedures, it has been shown that nodular lymphoma is a malignancy of human B lymphocytes and that poorly differentiated lymphoma, diffuse, is principally a malignancy of T lymphocytes. Finally, histiocytic medullary reticulosis appears to be a monocyte tumor. The procedures utilized here should be of importance in diagnosis, classification and prognosis of human lymphoid tumors. Moreover, as our knowledge of distinct metabolic and physiologic characteristics of lymphocyte subtypes improves, these diagnostic tools may allow rational therapeutic chaices to be made. (Green and Shevach, LI/NIAID, Frank, LCI, NIAID and Jaffe, LP, NCI).

Serial No. NIAID - 140
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Receptors on Immunocompetent Cells

Previous Serial No.: Same

Prinicpal Investigator: William E. Paul

Other Investigators: Project Subtitle I: Dr. F. Finkelman
Dr. E. Shevach
Dr. I. Green
Project Subtitle II: Dr. C.A. Janeway, Jr.

Cooperating Units:

Man Years:	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	3-5/12		2/12	3-7/12
Professional	2-10/12		2/12	3
Other	7/12			7/12

Project Subtitle I: Surface Components of Guinea Pig Lymphocytes.

Project Description:

Objectives:

The analysis of the chemical basis of antigen-recognition by lymphocytes and of the subsequent activation of these cells would be markedly aided by the isolation and characterization of the components of the lymphocyte membrane responsible for this recognition. The two principal classes of molecules which appear to be of importance in specific antigen-recognition are membrane associated immunoglobulin and the products of specific immune response (Ir) genes. Substantial work on the molecular characterization of membrane immunoglobulin of thymus-independent (B) lymphocytes of the mouse has been reported by several groups. Consequently, our studies of surface immunoglobulin of guinea pig B lymphoyctes were regarded as laying the groundwork for further studies of receptor structure. On the other hand, both the question of the existence of immunoglobulin on thymus-dependent (T) lymphocytes and of the structure of the Ir gene product are critical, and unresolved issues. In the latter case, study of guinea pig cells is particularly important as it only in this species that alloantisera capable of blocking

Ir gene function have been produced (see Project Report-142). These sera provide an important tool to characterize the products of genes closely linked to Ir genes and, quite possibly may allow an analysis of the Ir gene products themselves.

Methods Employed

Various cell types including lymph node lymphocytes, purified peritoneal exudate lymphocytes, peritoneal exudate cells and thymocytes from strain 2 and strain 13 guinea pigs and L_2C leukemia cells from strain 2 guinea pigs were radioiodinated in the living state by the lactoperoxidase procedure. This technique confines labelling to surface components and radioactive molecules can be regarded, in the main, to be membrane components. These components are extracted with the non-ionic detergent nonidet-P-40 (NP-40). The dialyzed extract is analyzed by sequential precipitation with characterized antisera. The immunoprecipitate is extensively washed, dissolved in sodium dodecyl sulfate (SDS) and subjected to electrophoresis in SDS-polyacrylamide gels.

Major Findings:

From the surface of strain 2 guinea pig lymph node cells, immunoglobulins with heavy (H) chains of mobility equal to that of authentic μ and γ chains are isolated. In general, the radioactivity assoicated with μ-like chains exceeds that assoicated with γ-like chains and the μ-like chains are found in relatively low molecular weight molecules, suggesting they exist as monomeric IgM. Whether the γ chains found on the surface of lymph node lymphocytes represent immunoglobulin synthesized by the cells which bear it or are cytophilic molecules has not yet been established.

After immunoglobulin has been specifically precipitated from the cell extract, antigens reacting with antisera directed at antigens coded for at the 2/13 locus can be precipitated by the addition of isologous anti-2 serum followed by rabbit anti-guinea pig immunoglobulin. The 2 alloantigen prepared in this way has a mobility on SDS-polyacrylamide gel electrophoresis consistent with a molecular weight of 25,000 daltons. No lower molecular weight chain has yet been isolated with the 2 alloantigen. Analysis of extracts of labeled strain 13 cells yields a product precipitable with anti-13 serum which has a molecular weight of 25,000 daltons. These molecular weights are quite different from those of the D and K antigens of mice and the LA and Four antigens of humans. Indeed, they resemble in molecular size the recently described Ia antigens of mice, which are the products of genes located in or very close to the region of the H-2 complex in which the great majority of most Ir genes are found. Furthermore, as described in Project 142, the 2/13 antigens and the Ia antigens are largely confined to the lymphoid tissue and anti-2 sera specifically block Ir gene product functions in a manner which

suggests a physical link between the 2 alloantigen and the 2-linked Ir gene products. Because of this, the molecule bearing the 2 alloantigen may also contain the Ir gene product. If the latter mediates a specific recognition function one would anticipate it to be highly heterogeneous molecule. Attempts to determine whether the 2 and 13 antigens display such molecular heterogeneity are now in progress.

Recently, a new series of guinea pig histocompatibility antigens have been described (see Project #142). The prototype antigen of this series, B, is present on a variety of tissues of both strain 2 and strain 13 guinea pigs. Anti-B sera identify an alloantigen in labelled extracts of strain 2 or strain 13 lymph node cells with a molecular weight of 45,000 daltons, associated with which is a second chain with a molecular weight of ∿ 12,000 daltons. The B antigen appears to be similar in this regard to the D and K antigens of mice and the LA and Four antigens of humans. Furthermore, the low molecular weight chain may be the guinea pig analog of β_2-microglobulin. Preliminary studies of anti-B alloantiserum indicate that it does not block antigen-recognition by T lymphocytes (Project 142), emphsizing the unique importance of the 2/13 alloantigens.

Significance to Biomedical Research and the Program of the Institute:

Ir gene products have a crucial role in antigen recognition by T lymphocytes. Indeed, in both experimental animals and humans, possession of given Ir genes confers susceptibility or resistance to given diseases to the individual. The delineation of the chemical basis for Ir gene product function may allow an understanding of how T lymphocytes recognize antigen and may lead to strategies for controlling Ir gene product function in clinically important situations.

Proposed Course of Project:

Efforts to further characterize the 2/13 antigens, to determine their molecular heterogeneity and to isolate them in larger yield are planned. In addition, use of the lactoperoxidase catalyzed iodination procedure will be made in attempts to label specific receptors of both T and B lymphocytes.

Project Subtitle II: Specificity of T cell Receptors for Antigen

Project Description:

Objectives:

T lymphocytes appear to play a central role in immune recognition and in the protective functions of the immune system. However, the nature of their surface receptor for antigen is unknown. The simplest

theory of immune recognition would have T cell receptors be immunoglobulin like molecules. There are two major problems with this theory. One is that surface immunoglobulin has not been reproducibly detected on T cell surfaces. The other is that the structures recognized by T cells appear to differ from those for which B cells or antibody are specific. This study was undertaken to evaluate a recent observation that T cells might, under certain conditions, recognize a chemically definable antigenic determinant, the 2,4 dinitrophenyl or DNP group. If this could be established, it would allow precise analysis of fine specificity of T cell receptors, particularly in comparison to that of anti-DNP antibody.

Methods Employed:

Peritoneal exudate lymphocytes from immune guinea pigs are composed largely of T cells, which will synthesize DNA in vitro when cultured in the presence of the antigen to which the donors of these cells have been immunized. Guinea pigs with T cell responses with apparent DNP specificity were prepared by immunization with DNP coupled directly to Mycobacterium tuberculosis of strain H37Ra (DNP-H37) and the responsiveness of their lymphocytes to a series of antigens was tested. Specificity of the antibody produced by such animals was determined by binding to radiolabelled antigens or by inhibition of binding to reference radiolabelled antigens.

Major Findings:

When guinea pigs are immunized with DNP-protein antigens, their peritoneal exudate lymphocytes (PELs) synthesize DNA in vitro when cultured with the immunizing DNP conjugate, or with the carrier alone. Little or no proliferation is observed when DNP conjugates of other proteins are placed in culture. This phenomenon is called carrier or conjugate specificity and is a well known property of T cells. On the other hand, priming with DNP-H37, while leading to PELs highly responsive to tuberculin, gives rise to a very good in vitro response to DNP coupled to a variety of protein carriers. Carrier alone fails to stimulate these cells nor do DNP-proteins stimulate cells from guinea pigs primed with H37 alone. Thus the hapten plays an essential role in the antigenic determinant these T cells recognize. This is further emphasized by studies using chemical analogs of DNP as hapten on a single type of carrier, the protein ovalbumin. These studies showed that the para-nitro group is essential both for T cell and for antibody recognition of the hapten. On the other hand, the ortho-nitro group plays little or no role in this response, nor is it necessary for antibody binding. The role of the linkage to the carrier protein in this response was elucidated by coupling DNP to protein by means of a flexible tripeptide spacer molecule, β-alanyl-glycyl-glycine, or DAG. DNP proteins are stimulatory, while DAG proteins are not. On the other hand, DNP and DAG proteins both bind antibody and participate in cell-cell collaboration in antibody responses; indeed, DAG proteins are more inhibitory of DNP binding than are DNP proteins. Thus, T cell recognition seems to involve precise recognition of the hapten determinant, plus additional determinants

provided by the carrier. Not only do DAG proteins fail to stimulate cells from DNP-H37 primed animals, but also DNP-Ficoll and DNP conjugates of synthetic copolymers of gultamic acid and lysine, DNP-L-GL and DNP-D-GL do not stimulate these cells. This failure to respond to DNP-L-GL was seen in both strain 2 and strain 13 guinea pigs, and therefore is not under the control of the Ir gene for DNP-L-GL. It was found that a carrier similar to L-GL, but containing 7% tyrosine, DNP-L-TGL, stimulated the T cells as well as DNP protein conjugates. Furthermore, removal of DNP groups from the tyrosine residues of L-TGL by selective thiolysis with 2-mercaptoethanol abolished stimulation by these compounds, although there were still as many DNP-lysine residues in the conjugate as before thiolysis. That the carrier had not simply been destroyed was shown by re-dinitrophenylation, which restored stimulatory capacity to DNP-L-TGL. Similar studies gave identical results using a terpolymer of tyrosine, glutamic acid and alanine, where the DNP will conjugate only to the N terminal amino group and to the hydroxy group of tyrosine. A number of attempts to inhibit this response with free hapten, including mono-O-DNP-tyrosine, have failed. Interestingly, pretreatment of the guinea pigs with DNP-D-GL can reduce their anti-DNP antibody response to DNP-H37 by 3000 fold without affecting their T cell response to hapten. This is further evidence for the distinction between T and B cell receptors for DNP. Extensive studies of the antibody produced by such animals has not yet revealed a serological correlate for the specificity of this T cell receptor.

In conclusion, these studies have defined in some detail the nature of the antigenic determinants recognized by these T cells. They recognize DNP with a high degree of precision, which correlates well with recognition of DNP by antibody from the same animal. However, T cells also recognize other determinants which are contributed by the carrier. Surprisingly, this determinant principally involves DNP linked to the hydroxy of tyrosine.

Significance to Biomedical Research and the Program of the Institute:

T cells are central to the precess of immune recognition, or self-non-self discrimination which is important in resistance to infection and to tumors, and in some forms of autoimmune disease. An understanding of how recognition takes place will be aided by precise definition of the specificity of the recognition site. The demonstration and evaluation of hapten specific T cells provides an important beginning to this analysis.

Proposed Course of Project:

The project will be pursued along several interrelated courses. 1. Further definition of T cell specificity using other haptens, other linkages to carrier, and other synthetic carriers. 2. Attempts are being made to extend the system to mice, where T cell subpopulations are

better defined. 3. A search will be made in this system for antigen binding T cells and, if found, they will be used for the study of inhibiiton of antigen binding by hapten. 4. An attempt will be made to design an affinity labelling reagent to specifically label this receptor and isolate it, so that it can be chemically characterized. 5. Using the knowledge provided by the exquisite sensitivity and specificity of T cells for chemically defined antigens, further attempts will be made to define precisely those determinants recognized by serum antibody and by B cells.

Publications:

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Serial No. NIAID - 141
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Lymphocyte Biology and the Interactions of Lymphocytes in the Immune Response

Previous Serial No.: Same

Principal Investigator: Dr. William E. Paul

Other Investigators:
Project Subtitle I: Dr. M. Gelfand, LI/NIAID
Dr. M. Frank, LCI/NIAID
R. Lieberman, LI/NIAID
Dr. D. Sachs, Immunology Branch, NCI
Project Subtitle II: Dr. M. Karpf, LI/NIAID
Dr. D.E. Mosier, LI/NIAID
Project Subtitle III: Dr. R. Sharon, Blood Bank Hebrew Univ. Jerusalem
Dr. P.R.B. McMaster, LMI/NIAID
Project Subtitle IV: Dr. C.A. Janeway, Jr., LI/NIAID

Cooperating Units: LMI/NIAID
LCI/NIAID
Immunology Branch, NCI

Man Years:

	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	4-7/12		4/12	4-11/12
Professional	2-2/12		4/12	2-6/12
Other	2-5/12			2-5/12

Project Subtitle I: Ontogeny of Bone Marrow Derived Lymphocytes

Objectives:

The development of the thymus independent (B) lymphocytes of mammals has been relatively difficult to study because no single central lymphoid organ in which B lymphocytes develop has yet been identified in mammals and because it is very difficult to obtain populations of B lymphocyte precursors devoid of mature B lymphocytes. As a possible solution to this difficulty, we have studied the events associated with the appearance of B lymphocytes in spleens and livers of neonatal mice.

We have previously demonstrated that lymphocytes bearing surface immunoglobulin increase markedly in number in the immediate post natal period and that the proportion of splenic lymphocytes with surface immunoglobulin in one week old mice approaches that of splenic lymphocytes of adult mice. On the other hand, B lymphocytes bearing receptors for the activated third component of complement (C3) are not detectable until later in life. This lag in the appearance of B lymphocytes with C receptors (CRL) as compared to Ig bearing B lymphocytes occurs in all mouse strains tested but is of very short duration in AKR mice, of intermediate duration in A mice and of long duration in many other strains, including C57BL/6, BALB/c and DBA/2. Our current experiments were aimed at determining whether this strain difference in the rate of CRL appearance was controlled by one or two genes or was dependent on the action of large numbers of genes.

Methods Employed:

Complement receptor lymphocytes were enumerated by the formation of rosettes with sheep erythrocytes (E) coated with 19S anti erythrocyte antibody (A) and complement (C). Mice to be tested for frequency of CRL were 12 to 17 days of age, although efforts were made to use animals that were precisely two weeks old.

Major Findings:

Analysis of CRL frequency in 2 week old AKR, DBA/2, (AKR x DBA/2)F_1 (F_1 x AKR) and (F_1 x DBA/2) mice indicated that AKR mice differed from DBA/2 mice at two genes important in determining CRL frequency in young mice. In particular, 2 week old F_1 mice have a CRL frequency intermediate between that of AKR (high) and DBA/2 (low). Of the progeny of an F_1 x DBA/2 cross, approximately 25% resemble the DBA/2 parent and have few CRL at two weeks of age, while the remainder have intermediate or high CRL frequency. When the histocompatibility type of low CRL mice of this backcross is determined, all are found to be homozygous for the histocompatibility type of the low CRL parent (DBA/2), indicating that one of the genes involved in controlling the rate at which CRL appear is linked to the major histocompatibility complex. This conclusion is supported by the frequency of CRL in two week old H-2 congenic mice. Thus, B10.A mice have an intermediate CRL frequency like A mice, which are the donors of the H-2 region of the B10.A, but unlike B10 mice, which donated the remainder of the genome to the B10.A. Furthermore, two other B10 congenic strains are of low CRL type (B10.BR and B10.D2). In another congenic series, the A.By, A.Ca and A.Sw are all of low CRL type, while, as noted above, the A is intermediate.

We have termed the H-2 linked gene involved in the control of CRL development CRL-1 and by study of H-2 and H-2-Tla recombinant mice, have localized CRL-1 to the right of the genes controlling serum substance

(Ss-Slp) and to the left of the genes controlling the TL antigens. To our knowledge this is the first H-2 linked gene controlling B lymphocyte development which has been identified.

One additional study which the time table of CRL appearance in neonatal mice makes possible is an evaluation of the role of the complement receptor in B cell activation. One hypothesis, advanced by Dukor and Hartmann, is that the activation of B lymphocytes requires the interaction of activated complement with the C3 receptors on the B cell surface. If this thesis were correct, then B lymphocytes which lack complement receptors should fail to respond to agents which stimulate mitosis in B lymphocytes. Such populations of complement receptor negative B lymphocytes may be obtained from the spleens of 3 to 5 day old BALB/c mice. These spleen cells, although lacking detectable CRL, respond very vigorously to the B cell mitogens E. coli lipopolysaccharide and poly inosinic . poly cytodilic acid. This result indicates that the complement receptor of the B lymphocyte is not an absolute requisite for the activation of that cell.

Significance to Biomedical Research and the Program of the Institute:

These studies provide us with cellular and genetic tools to more precisely analyze the process of development of B lymphocytes and the mechanisms by which these cells are activated. As this cell type is responsible for the production of antibodies, this regulatory information may be of importance in attempts to manipulate B cell functions more precisely.

Proposed Course of Project:

Principal emphasis will be placed on developing methods for assessing molecular requirements for B cell activation and tolerance induction.

Project Subtitle II: Responses of B lymphocytes to "T-dependent" antigens in the absence of T lymphocytes

Obectives:

A complete humoral immune response to most antigens requires the collaborative function of T lymphocytes in the activation of precursors of antibody secreting cells. The role of "helper" T cells in B cell activation is still not clear. Indeed, it is not certain whether the principal role of the T cell is to present antigen to the B cell or to regulate the response of the B cell after it has been activated by antigen. If the latter hypothesis is correct, then one would anticipate that, even in the complete absence of T cells, so-called "T-dependent" antigens should cause some type of response on the part of B cells. Indeed, in last year's annual report we presented results based on

immunization of thymus-deprived mice which support this view. In order to investigate this issue more thoroughly, we tested the in vitro response of spleen cells of normal and athymic mice to T-dependent antigens.

Methods Employed:

Spleen cell suspensions from normal and congenitally athymic (nu/nu) mice were cultured in Microtiter plates in the presence of various stimulants including E. coli lipopolysaccharide (LPS), 2,4-dinitrophenyl (DNP) hemocyanin or DNP-bovine serum albumin (DNP-BSA). At various times after initiation of culture (usually 72 hours) incorporation of tritiated thymidine (3H-TdR) into DNA was determined. In addition, synthesis of immunoglobulin (Ig) was measured in some cultures by determining incorporation of 3H-leucine into material which was precipitable with anti-Ig.

Major Findings:

Both DNP-BSA and DNP-hemocyanin proved capable of initiating DNA synthetic responses on the part of spleens from normal and DNP-primed animals. The responses were of the order of 6 to 10 fold in magnitude, were not diminished by pretreatment of spleen cells with anti- and C and could be obtained with cells from congenitally athymic (nu/nu) mice. The responses did not appear to be due to contamination with endotoxin as many of the DNP-proteins were highly stimulatory even when LPS contamination was less than 1 part in 10^{+6}. Moreover, responses to both DNP-hemocyanin and DNP-BSA were inhibited by the presence of purified anti-kappa serum (without complement) whereas the response to LPS was not affected by anti-kappa. This further supports the contention that DNP-BSA and DNP-hemocyanin stimulation is not a function of LPS contamination and suggests that the DNP-proteins interact with specific receptors on the B cell surface

Since DNP-BSA is a classical "T-dependent" antigen, we anticipated that the response observed to it on the part of spleen cells from nu/nu mice should be incomplete. Indeed, we found that whereas LPS and DNP-hemocyanin stimulated spleen cells from nu/nu mice to synthesize DNA and immunoglobulin, DNP-BSA cause little Ig synthesis in comparison to the DNA synthesis it initiated.

This result strongly suggests that T lymphocytes mediate their helper function not by allowing B cells to recognize antigen but rather by controlling the differentiation of the B cell after it has interacted with antigen.

Significance to Biomedical Research and the Program of the Institute:

These studies help to explain the role of T lymphocytes in regulating responses of B lymphocytes and provide some insights into the molecular structures required for B cell activation. They should provide valuable information in delineating mechanisms to control magnitude and type of in vitro antibody responses.

Proposed Course of Project:

We anticipate a more thoroughgoing evaluation of the conditions under which DNP-BSA leads to B cell activation and in particular we expect to undertake a detailed study of molecular requirements for this activation.

Project Subtitle III: Activation of B lymphocytes by DNP-Ficoll

Project Description:

Objectives:

Immune responses to thymus (T)-independent antigens appear to differ in several critical ways from immune responses to T-dependent antigens. In particular, responses to DNP-derivatives of several polysaccharides tend to yield responses in which IgM antibody dominates and in which little or no immunologic memory can be demonstrated. Recently, Philip McMaster (LMI/NIAID) prepared a DNP-derivative of Ficoll, a commercial polymer of sucrose and epichlorhydren. We have found this antigen particularly valuable in attempting to evaluate the capacity of T-independent antigens to stimulate IgG responses and in the ability of these antigens to generate and stimulate DNP-specific memory cells.

Methods Employed:

The immune response to DNP-Ficoll has been evaluated by in vitro culture techniques (see serial No. 144) and by immunization of normal and congenitally athymic (nu/nu) mice with varying doses of DNP-Ficoll dissolved in physiologic saline. The immune response is measured in terms of numbers of spleen cells capable of forming plaques with dinitrophenyl (DNP) or trinitrophenyl (TNP) derivatized erythrocytes. In many experiments, the response of cells was assayed by transferring graded numbers of spleen cells to irradiated recipients which were then immunized.

Major Findings:

Doses of DNP-Ficoll of from 1 to 1000 ug elicit substantial anti-DNP responses in many strains of mice. The optimal response is observed

to 100 ug of DNP-Ficoll at 7 days after priming. At this time, ∿100,000 IgM DNP-specific plaque forming cells (pfc) per spleen are found in C57BL/6 mice. This response is independent of thymus-dependent (T) lymphocytes as equivalent responses are obtained in congenitally athymic (nu/nu) mice and their littermate (+/nu and +/+) controls. In addition, considerable numbers of IgG_2 (∿50,000/ spleen) and IgG_1 (∿25,000/spleen) pfc are observed both in normal and athymic mice. This finding suggests that DNP-Ficoll is a particularly interesting antigen, as the majority of T-independent antigens have been reported to elicit little or no antibody of the IgG class. One possible reason for this difference is that Ficoll is a much more highley cross-linked substance than most other T-independent antigens. To test this, we intend to prepare DNP-Ficoll derivatives of varying degrees of size and cross linkage by controlled acid hydrolysis and enzymatic digestion. A second interesting feature of the response to this agent is its failure to yield secondary responses. This is true, thus far, in three different types of situations. Firstly, priming with DNP-Ficoll and challenging with DNP-Ficoll at varying times does not cause a secondary response. Secondly, priming with DNP-proteins does not prepare for a secondary response to DNP-Ficoll and thirdly, priming with DNP-Ficoll and challenging with DNP-ovalbumin, in the presence of ovalbumin specific helper cells, does not yield a secondary anti-DNP response. These results suggest that priming with DNP-Ficoll does not expand the DNP-specific pool of responsive cells and raise the possibility that B lymphocytes responsive to T-independent antigens are distinct from B lymphocytes responsive to T-dependent antigens. Studies are now in progress to establish whether DNP-Ficoll treatment renders mice specifically unresponsive to DNP proteins.

Significance to Biomedical Research and the Program of the Institute:

A large number of clinically important antibody response are directed to highly polymeric polysaccharide antigens. These responses are different from the more thoroughly evaluated responses to protein and cellular antigens and their regulation is poorly understood. DNP-Ficoll appears to be an antigen which will allow a considerable delineation of the control of these responses in mice and may provide insight into ways to manipulate such responses in humans.

Proposed Course of Project:

We intend to delineate in more detail the tolerogenic capacity of DNP-Ficoll, to evaluate the cytokinetics of the response to this agent and by use of structural analogs, to attempt to determine whether a precise array of antigenic determinants is the key feature in the capacity of DNP-Ficoll to activate B lymphocytes directly.

Project Subittle IV: Immunocompetent cell interactions in antibody responses

Objectives:

Antibody responses to many antigens require the cooperative interaction of two classes of lymphoid cells. These classes of lymphocytes are the thymus independent (B) lymphocytes and the thymus dependent (T) lymphocytes. Both cells have specificity for antigen, the B cell via antibody-like receptors on its surface, the T cell via an uncharacterized receptor. The mode of communication between these two cells is not yet fully understood. These cell types can be readily examined by means of adoptive transfer experiments of immune cells into irradiated syngeneic recipients. The objectives of these studies have been: 1. To gain an understanding of the process by which T helper cells recognize antigen; 2. To develop means for selectively effecting the activity of T or B cells; 3. To understand the process of cell-cell interaction in the antibody response; 4. To define the role of serum antibody in the regulation of the immune response.

Methods Employed:

Irradiated mice, or mice treated with various agents, are reconstituted with cells from normal or immunized syngeneic mice. Passively administered antibody is used in some experiments. The recipient mice are boosted with antigen. The subsequent antibody response is a complex function of the activity of the interacting cell types.

Major Findings:

One approach to the analysis of the functional characteristics of specific T and B lymphocytes involves the transfer of T lymphocytes from a donor primed with a carrier protein and B lymphocytes from a donor primed with a hapten conjugated to a different carrier. The recipients are then challenged with a conjugate of the hapten to the first protein. By holding the hapten-specific (B) cells in constant and excess numbers, one can evaluate the effect of varying the number of transferred carrier specific (T) cells. Alternatively, if a constant and excess number of T cells are transferred, the effect of varying B cell number may be evaluated. Such titrations reveal a linear relation between B cell number and antibody response. However, an exponential relation between antibody response and T cell number exists, in which the response is proportional to the square of the number of T cells administered. Such a result is consistent with the existence of two interacting T cell types, both of which are present in limiting numbers. However, a variety of other explanations are possible including increased antibody production resulting from 2 interactions of one B cell with T cells of the same type. A series of attempts to demonstrate two types of T cells have been mode. These have included the use of graded doses of x-irradiation, administration of anti-thymocyte serum (ATS) and adult thymectomy. No evidence for T cell interaction in the helper effect has been obtained but no other explanation for this effect has yet been demonstrated.

A second major problem explored in these experiments is the role of antibody in augmenting immune responses to very weak antigens. We have previously demonstrated that the 2,4-dinitrophenyl derivative of the BALB/c IgG myeloma protein LPC-1 fails to elicit an antibody response to the LPC-1 idiotype in BALB/c mice. However, if small amounts of anti-DNP antibody are administered together with DNP-LPC-1, a clear anti-idiotype response ensues. A major question regarding these experiments is the importance of T cell participation in the response of the host to this immunization regimen. We found that mice deprived of T lymphocyte function by a series of strategies are unresponsive to DNP-LPC-1, even if administered with anti-DNP antibody. However, reconstitutuion of such animals with small numbers of splenic T lymphocytes allows them to respond to DNP-LPC-1 and anti-DNP antibody. This suggests the possibility that antigen-antibody complexes may, under certain circumstances, be highly immunogenic and may achieve this by non-specifically activating T lymphocytes.

Significance to Biomedical Research and the Program of the Institute:

The T lymphocytes have a critical role in the regulation of both normal and abnormal immune responses. In order to control these responses, a precise understanding of the mode of function of these cells is required. Furthermore, the experiments suggesting that antigen-antibody complexes may activate T lymphocytes have considerable importance in increasing immune responses to weak antigens such as tumor specific antigens.

Proposed Course of Project:

There are several major areas that need further exploration. In particular, the elucidation of the interacting helper cells, the further understanding of the role of antibody in augmenting immune responses, the nature of the T cell receptors for antigen and for antigen-antibody complexes, and the role of regulatory or suppressor T cells will be pursued.

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Serial No. NIAID - 142
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Genetic control of immune response

Previous Serial No.: Same

Principal Investigator: Dr. Ethan Shevach and Dr. Ira Green

Other Investigators: Project Subtitle I: Dr. William E. Paul, LI/NIAID
Dr. Z. Ben-Sasson, LI/NIAID
Project Subtitle II: Dr. William E. Paul, LI/NIAID
Dr. Fred Finkelman, LI/NIAID
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Dr. G. Forni, LI/NIAID
Dr. A. DeWeck, Inselspital, Bern Switzerland
Dr. A. Geczy, Inselpital, Bern Switzerland
Project Subtitle III: Dr. A. Rosenthal, LCI, NIAID
Dr. P. Lipsky, LCI, NIAID

Cooperating Units:

Man Years:	NIAID Lab Staff	Other NIH Personnel Assigned to	Guest Workers	TOTAL
Total	4		1/12	4-1/12
Professional	2-6/12		1/12	2-7/12
Other	1-6/12			1-6/12

Project Subtitle I: Effect of Alloantisera on Immune Response Gene Product Function

Project Description:

Objectives:

A series of genes have been described in guinea pigs, mice, rats and in man which control the capacity to mount specific immune responses. One important group of immune response (Ir) genes is linked to the major histocompatibility (H) complex of the species. These H-linked Ir genes have been demonstrated to function mainly in the thymus derived (T) lymphocyte. However, the mechanism by which H-linked Ir genes and their products control immune responses is still unknown. We have demonstrated that alloantisera prepared by cross immunizing strain 2 and strain 13 guinea pigs with lymphoid

cells markedly inhibit the in vitro proliferative response to antigens the response to which is controlled by the Ir gene which is linked to the H antigen against which the serum is directed. Thus, when the T lymphocytes from (2 x 13)F_1 guinea pigs are cultured in vitro, anti-2 serum specifically inhibits the proliferative response to the 2,4-dinitrophenyl derivative of a copolymer of L-glutamic acid, L-lysine (DNP-GL, an antigen the response to which is controlled by a 2-linked Ir gene) while anti-13 serum inhibits the in vitro proliferative response to a copolymer of L-glutamic acid and L-tyrosine (GT, an antigen the response to which is controlled by a 13-linked Ir gene). We concluded from this observation that the Ir genes produce a cell surface product which plays a role in antigen recognition and whose function can either directly or indirectly be blocked by alloantisera. Experiments were therefore designed to determine the precise target (H antigen or Ir gene product) against which the alloantisera exert their inhibitory effect and to determine the precise mechanisms by which alloantisera block T cell antigen recognition.

Major Findings:

The most likely mechanism for the inhibition of Ir gene product function by anti-H sera is that the Ir gene products and the H antigens are physically linked or close together on the cell surface. Alternatively, the alloantisera might contain in addition to anti-H antibodies, antibodies directed against and capable of directly blocking the specific Ir gene products. In order to distinguish between these two alternatives it would be advantageous to analyze the ability of alloantisera to inhibit T cell proliferation to an antigen, the response to which is under control of a specific Ir gene, in the absence of the H antigen which usually occurs in association with that Ir gene. However, in progeny of crosses of (2 x 13)F_1 and parental animals we have not observed a recombinant chromosome in which a given Ir gene has been separated from its linked H-antigen gene. We therefore have utilized outbred animals in which relationships between H genes and Ir genes exist which differ from those in inbred populations. In inbred animals the Ir gene which controls responsiveness to a copolymer to L-glutamic acid and L-alanine (GA) is linked to the poly-lysine (PLL) Ir gene and to the strain 2 H-antigen genes. In some outbred animals the GA gene is also associated with the PLL gene and the 2-H antigen gene. However, in other outbred animals the GA Ir gene can be found in the absence of both the PLL gene and the strain 2 H-antigen gene. We have used this group of $GA^+PLL^-2^-$ animals in an attempt to demonstrate whether anti-2 serum can inhibit the function of the GA gene when this gene occurs in an animal lacking the strain 2 H-antigen genes. The results of this study demonstrated that anti-2 serum was only capable of inhibiting the in vtiro proliferative response to GA of cells derived from $GA^+PLL^+2^+$ animals; anti-2 serum had no effect on the proliferative response to GA of cells derived from $GA^+PLL^-2^-$ animals. This result suggests that the blocking activity of the anti-2 serum is mediated by antibodies specific for the strain 2 alloantigen and not by antibodies directed against the GA Ir gene product.

Another approach to the analysis of the target antigens of the alloantisera is the preparation of a serum which might be specific for the GA Ir gene product and be free of antibodies to the strain 2 alloantigen. We therefore immunized strain 13 guinea pigs with the lymphocytes of $GA^{+}PLL^{-}2^{-}13^{+}$ animals; another group of strain 13 animals were immunized with the cells derived from $GA^{+}PLL^{+}2^{+}13^{+}$ animals. In the former case the resultant sera would be free of anti-strain 2 antibodies, while in the latter immunization the sera should contain antibodies to the strain 2 alloantigen. When these sera were tested for their ability to inhibit the GA response of cells derived from $(2 \times 13)F_1$ guinea pigs immune to GA, only the 13_{+}anti-$GA^{+}PLL^{+}2^{+}13^{+}$ serum inhibited the GA response. The 13 anti-$GA^{+}PLL^{-}2^{-}13^{+}$ serum was devoid of inhibitory effect. This study again indicates that the target antigen against which the inhibitory activity of the anti-2 serum is directed is the strain 2 alloantigen rather than the GA Ir gene product. Thus far, all attempts to prepare an alloantiserum which would be specific for a given Ir gene product have failed.

The studies described above suggest that the proposed mechanism whereby alloantisera mediate their suppressive effect on Ir gene product function, namely indirect blockade of the Ir gene products by antibodies directly reactive with H antigens, requires phenotypic linkage of the products of independent but closely linked genes. Recent studies of the lymphocyte membrane indicate that individual protein molecules are capable of free diffusion in the plane of the membrane; in order for two determinants to maintain the relationship required for an indirect (or steric) blockade a physical linkage between the two moieties would probably be necessary. Without such a link, the molecules would be separated in the course of free diffusion. One possible model for the relationship between the 2/13 antigens and the Ir gene products is that the genes for these structures, although distinct from each other produce a single polypeptide chain. In this model, 2/13 antigen part of the chain would be analogous to the constant region of an immunoglobulin heavy chain, while the specific Ir gene products would be analogous to the variable portion of an immunoglobulin heavy chain. Furthermore, the 2/13 antigen would be the same on all cells while an individual Ir gene product would have a clonal distribution. The exact functional role of this proposed 2/13 - Ir complex is not known; it might function as the specific antigen binding receptor of the T lymphocyte for it might function as an auxillary receptor and act in concert with a specific T cell immunoglobulin receptor.

Although we have demonstrated that the alloantisera act in a specific fashion in their inhibition of T cell proliferation in the $(2 \times 13)F_1$ animal, one possible explanation for this apparent specific inhibition would be that among the population of $(2 \times 13)F_1$ lymphocytes those responsive to DNP-GL would exclusively or predominantly express the strain 2 alloantigen while those responsive to GT would show a similar

preference in the expression of strain 13 alloantigens. If this explanation were true, then alloantiserum induced blockade could be explained by a general inactivation of the responsive cell. We therefore sought by a variety of means to establish whether or not allelic exclusion exists for the expression of the products of the 2/13 alloantigen genes. Using indirect immunofluorescence to evaluate (2 x 13)F_1 lymphocytes we found that greater than 95% of (2 x 13)F_1 T lymphocytes can be stained by anti-2 serum alone, anti-13 serum alone or anti-2 plus anti-13 serum. Similarly, the same degree of complement mediated lysis of chromated (2 x 13)F_1 lymphocytes is achieved by anti-2 serum, anti-13 serum or a mixture of the two. Finally, if (2 x 13)F_1 lymphocytes are treated with anti-2 serum and complement at a dilution that does not give 100% lysis, the cells remaining alive do not show a preferential diminution in their responsiveness to DNP-GL as compared to their responsiveness to GT. This suggests that the T lymphocytes richest in strain 2 alloantigen are not more likely to respond to DNP-GL than to GT. Thus, we have found no evidence for the preferential expression of the 2 or 13 alloantigens on a subpopulaiton of (2 x 13)F_1 lymphoid cells. Therefore it appears most likely that the 2/13 antigens are either physically linked to the Ir gene products or represent constant parts of a 2/13 -Ir polypeptide chain as discussed above.

It has been implicit in the experiments described thus far that alloantisera block the <u>in vitro</u> proliferative response to antigen by either directly or indirectly interfering with the interaction of antigen and immune T lymphocytes. However, as yet we have no direct evidence that alloantisera inhibit antigen recognition and not some later event in the <u>in vitro</u> proliferative response to antigen. In order to examine this question in greater detail, we have performed time course studies with the alloantisera. If the alloantisera inhibit antigen recognition then pretreatment of T lymphocytes with the sera and/or the use of the antisera early in the 3 day culture period followed by its removal and replacement with normal serum should still produce significant inhibition of cell proliferation. The results of this study demonstrated that pretreatment of T lymphocytes for as long as 24 hours with the alloantisera had no effect on their subsequent response to antigen. Of greater interest was the observation that adding the serum for the initial 24 hour period of exposure to antigen followed by washing and replacement with normal serum had no effect on cell proliferation, while addition of the alloantiserum after 24 hours of culture in normal serum still produced 80-90% of the maximun inhibitory effect. This study raises the possibility that the alloantisera inhibit cell proliferation by acting at some later stage beyond the initial antigen recognition event; however, it is also possible that cell proliferation as measured over a 72 hour period <u>in vitro</u> requires repeated exposure of the immune T cells to antigenic stimulation. In order to resolve this question it is necessary to obtain antigen stimulated T lymphocytes free of the stimulating antigen. This problem has proven difficult to resolve because the antigen present in these cultures is bound to the surface of cells and cannot be removed by

simple washing procedures. One approach we have utilized is to allow antigen pulsed macrophages to adhere to the surface of Petri dishes followed by the addition of immune T lymphocytes for varying periods of time. Since the latter population does not adhere to the dishes, we are able to study their proliferative response following removal from the dishes both in the presence and absence of additional antigen. If the T cells are allowed to remain on the dish for 24 hours, removed and then cultured for 72 hours, a small proliferature response is observed. This response is approximately 1/10 of the response seen when antigen is added to the same population of cells. We observed that anti-2 serum is capable of blocking the stimualtion of cells produced by a 24 hour incubation on the dish when it is present during the incubation period on the Petri dish; however, anti-2 serum also inhibited DNA synthesis when it was subsequently added to the culture tube. This result suggests that the maximum in vitro DNA synthetic response to antigen requires the constant presence of antigen and that anti-2 serum is indeed capable of blocking if present early in the culture period during the antigen recognition phase. The mechanism of the blockade of cell proliferation produced by anti-2 serum when it is added after 24 hours in the absence of added antigen is still under investigation.

Significance to Biomedical Research and the Program of the Institute:

The chemical nature of the antigen receptor of the T lymphocyte is unknown at the present time. If the Ir gene product itself is not the prime receptor of the T lymphocyte, it is likely that the Ir gene product plays a significant auxiliary role in T lymphocyte antigen recognition. It is likely that further study and characterization of the Ir gene product will aid in the elucidation of the mechanism of T lymphocyte function in the regulation of the normal immune response. Since susceptibility to a number of malignant, auto-immune, and allergic diseases has been linked to histocompatibility type, Ir genes may play an important role in susceptibility to a wide variety of diseases.

Proposed Course of Project:

There studies will be further prusued with the major goals being development of sera which would facilitate the isolation and chemical characterization of the Ir gene products. The possible functional relationship between Ir gene products and another class of receptors (for example, immunoglobulin) on the T cell will also be investigated.

Project Subtitle II; Serologic Characterization of Guinea Pig Histocompatibility Antigens

Project Description:

We have presented experimental evidence that the target antigens against which alloantisera exert their inhibitory effect on T cell proliferation are coded for by the 2/13 alloantigen genes. It has also been suggested that the 2/13 antigens and the Ir gene products are in some manner phenotypically linked on the surface of the T lymphocyte. Although the Ir gene products are linked to 2/13 antigens it is unkown whether the latter are the equivalents of the well defined serologically detectable histocompatibility (H) antigens in the mouse (the D or K antigens) or in man (the LA or 4 antigens). In order to further our understanding of the mechamism of action of the Ir genes it has been necessary to further our understanding of the guinea pig H antigens. A system of guinea pig H antigens has been developed by a group of investigators working at the Institute for Clinical Immunology in Bern, Switzerland. By cross-immunizing outbred guinea pigs these investigators defined a single locus (or a group of closely linked loci) with 3 major alleles. This locus has been termed the GPL-A locus. We have carried out collaborative studies with the group in Bern in order to better characterize the GPL-A locus and its relationship to the locus controlling the 2/13 antigens. In addition, we have compared the biological and chemical characteristics of the antigens coded for by these 2 loci.

Major Findings:

Antisera to the three will defined alleles of the GPL-A locus (B,C,D,) were obtained from the group in Bern. Initial studies confirmed their earlier work that NIH strain 2 and strain 13 animals both bear the B antigens and are negative for the C and D specificities. Screening studies of the outbred guinea pig population available at the NIH revealed no association between the GPL-A locus, the 2/13 locus, and the PLL, GA, or GT Ir genes. Certain samples of antisera obtained form the Swiss group which were said to be monospecific for alleles of alleles of the GPL-A locus were found to be contaminated with antibodies directed against the 2/13 antigens. By using the NIH strain 2 and 13 animals as recipients we were able to prepare highly potent and specific sera to products of alleles at the GPL-A locus which were free of anti-2 or anti-13 activity. During the course of these studies we have discovered at least 2 more alleles at the GPL-A locus and in addition we have preliminary evidence for another locus which appears to be distinct from both the GPL-A locus and the 2/13 locus.

We next determined the relationship between the 2/13 antigens and the B antigen. One possible explanation for the results observed is that the B antigen represents a "public" specificity and the 2/13 antigens represent "private" specificities of a single H locus. By using the immunofluorescent technique we studied the relationships of the 2/13 antigens and the B antigen on the cell surface. The results demonstrated that the 2/13 antigens and the B antigen move independently and aggregate

or cap independently on the cell surface. This indicates that the 2/13 antigens and the B antigen are independent molecules on the cell surface and not 2 determinants on the same polypeptide chain. In order to show that the 2/13 antigens and the GPL-A antigens were both products of genes in the major histocompatibility complex, breeding studies to demonstrate linkage were undertaken. Although only a small number of offspring have as yet been analyzed, the preliminary results are highly suggestive that the 2/13 locus is linked to the GPL-A locus.

In most species that have been analyzed, 2 major loci controlling serologically defined H antigens have been identified. One possibility is that the 2/13 locus represents one of these loci and the GPL-A locus represents the other major locus in the guinea pig. However, a comparison of the tissue distribution of the 2/13 antigens and the B antigen suggests that this explanation is unlikely. The B antigen can be demonstrated both on lymphoid cells and on a number of non-lymphoid tumors such as a hepatoma and a fibrosarcoma. In contrast, the 2/13 antigens can only be identified on B lymphocytes, T lymphocytes, and cells of the monocyte-macrophages series. We have not been able to demonstrate the 2/13 antigens on a number of non-lymphoid cells either by absorption or by the sensitive indirect immunofluorescent technique. Furthermore, the 2/13 antigens are represented to a greater extent on the B lymphocyte than the T lymphocyte. Because of the profound effect of anti-2 serum on the proliferation of strain 2 lymphcoytes to any antigen, it was of interest to study the effect of anti-B serum on the in vitro proliferative response of strain 2 and strain 13 lymphoid cells. In a small number of preliminary studies, anti-B serum had little if any effect on the proliferative response of strain 2 cells to DNP-GL or to tuberculin (PPD) while anti-2 serum caused a marked inhibiiton of both the DNP-GL and PPD responses. In similar fashion anti-13 serum inhibited the response of strain 13 cells to GT and PPD while anti-B serum was devoid of inhibitory effect. Thus, the 2/13 antigens do not appear to be the equivalent of the D or K antigens in the mouse or the LA or 4 antigens in man. They have a restricted tissue distribution and antisera directed towards what appear to be the guinea pig equivalent of a major histocompatibility locus (the GPA-A locus) are without inhibitory effect on T cell proliferation.

Significance to Biomedical Research and the Program of the Institute:

The guinea pig is widely used in immunologic research for studies of delayed hypersensitivity and genetic control of the immune response. These studies have been hampered by our lack of knowledge of guinea pig histocompatibility antigens. The development and refinement of a system of histocompatibility antigens in the guinea pig will make this animal an excellent subject not only for research on genetic control of the immune response but also for transplantation tumor immunology. Further use of the guinea pig for studies in these areas should be of great importance as the immunologic makeup of the guinea pig closely resembles that of man.

Proposed Course of Project:

These studies will be pursued with the major goals being the understanding of the major histocompatibility antigens in the guinea pig and the relationship between these antigens and the products of the immune response genes. An attempt will also be made to develop new inbred strains of guinea pigs.

Project Subtitle III: Role of the macrophage in genetic control of the Immune Response

Project Description:

Previous studies have demonstrated that in order for macrophages to activate T lymphocytes to proliferate *in vitro* both cell types must be histocompatible. Thus, macrophages obtained from strain 2 guinea pigs and pulsed with antigen fail to activate immune strain 13 T cells to proliferate. It was also noted that macrophages obtained from an animal which lacked a given Ir gene (non-responder) were much less effieient in the induction of T cell proliferation in the F_1 (non-responder x responder) animal than macrophages obtained from the responder parent. These studies suggest that macrophage-lymphocyte interaction is mediated by either H antigens themselves or by the products of genes closely linked to the H antigen genes. We have extended these studies in an attempt to correlate functional macrophage-lymphocyte interaction with morphological studies of macrophage-lymphocyte interaction.

Major Findings

Physical interaction between lymphocytes and macrophages is observed during cultivation of these cells. Using an *in vitro* method to examine the binding of non-adherent lymphoid cells to macrophages cultured in microscope tissue culture chambers, the number of lymphocytes bound to macrophages can be counted and the degree of binding quantitated. If the macrophages are not pulsed with antigen and the degree of binding is quantitated at one hour after addition of the lymphocytes, no significant difference is observed in the binding of immune lymphocytes to either syngeneic or allogeneic macrophages. This suggests that the initial antigen-independent binding is not mediated via the 2/13 alloantigens or related structures. In contrast, if the macrophages are pulsed with antigen and the time of *in vitro* exposure to immune T lymphocytes prolonged to 24 hours, there is a marked increase in lymphocytes bound/100 macrophages only if macrophages and lymphocyte are syngeneic. This 24 hour antigen dependent binding was not observed during *in vitro* culture when immune lymphocytes were exposed to antigen-pulsed allogeneic macrophages. These results indicate that not only the functional but also the physical interaction of macrophages with immune T lymphocytes is related to the 2/13 alloantigens or products of genes closely linked to the alloantigen genes.

Significance to Biomedical Research and Program of the Institute:

The role of the macrophage in the immune response is not clearly defined. A number of studies indicate that the macrophage is required for the specific activation of immune T lymphocytes. The studies described here demonstrate that the macrophage also plays a role in genetic control of the immune response and that alloantigens or the Ir gene products themselves are important in cell to cell interaction. As recent studies have also demonstrated the importance of H antigens in the interaction of T and B lymphocytes, the methods developed for direct observation of the morphological events in macrophage-lymphocyte interaction may be very useful in the understanding of the way cells interact in the generation of the immune response.

Proposed Course of Project:

These studies will be continued and the role of alloantigens in macrophage-lymphocyte interaction further refined by the use of specific alloantisera in an attempt to block the physical interaction of macrophage and lymphocyte. The possible role of the GPL-A antigens in macrophage-lymphocyte interaction will also be examined.

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Rosenthal, A.S., Lipsky, P.E., and Shevach, E.M. Macrophage-lymphocyte interaction: Morphologic and functional correlates. In The Mononuclear Phagocyte, edited by R. VanFurth, in press.

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Serial No. NIAID - [illegible]
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1973

Project Title: Lymphocyte Interactions, Receptors and Functions

Previous Serial No.: Same

Principal Investigator: Dr. Ira Green

Other Investigators:
Project Subtitle I: Dr. Ethan Shevach, LI/NIAID
Dr. Michael Frank, LCI/NIAID
Dr. Elaine Jaffe, LP/NCI

Project Subtitle II: Dr. Richard Edelson, D/NCI
Dr. Ethan Shevach, LI/NIAID
Dr. Michael Frank, LCI/NIAID
Dr. Charles Kirkpatrick, LCI/NIAID
Dr. Takeshi Yoshida, Dept. of Pathology, St. Univ. of N.Y.
Dr. Stanley Cohen, Dept. of Pathology, St. Univ. of N.Y.
Dr. Marvin Lutzner

Project Subtitle III: Dr. Marshal Sklar, LVD/NIAID
Dr. Ethan Shevach, LI/NIAID
Dr. Michael Potter, LCBGY/NCI,
Dr. Richard Asofsky, LMI/NIAID

Cooperating Units: Dermatology Branch, NCI. Hematopathology Section, Laboratory of Pathology, NCI. Laboratory of Clinical Investigation, NIAID. Laboratory of Microbiol. Immunity, NIAID. Laboratory of Cell Biology, NCI. Laboratory of Viral Diseases, NCI. Department of Pathology, State Univ. of New York at Buffalo, Buffalo, New York.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to	Guest Workers	TOTAL
Total	2-3/12		3/12	2-6/12
Professional	9/12		3/12	1
Other	1-6/12			1-6/12

Project Subtitle I: Identification of the origin of human malignant reticular cells by the use of surface membrane markers

Project Description:

Objectives:

Cells involved in the immune response can be functionally characterized by the presence of different membrane antigens and receptors. In the mouse, thymus-dependent (T) lymphocyte can be identified by the presence of the θ isoantigen; in man, T cells have been found to form rosettes with sheep red blood cells (E). Thymus-independent (B) lymphocytes in most species studied, can be identified by the presence of easily detectable surface immunoglobulin and a receptor for antigen-antibody complement complexes (EAC). Monocytes and macrophages, the third cell type involved in the immune response, also bear a receptor for EAC, but they can be distinguished from B cells because they also bear a receptor for antigen-antibody-complexes (EA). The presence or absence of these various receptors on cells of unknown type may provide information as to their origin. In this study, malignant lymphoid cells from humans were examined for the presence of these various receptors.

Methods Employed:

In order to detect the EAC receptor, the lymphoid cells to be studied are incubated with red cells that have been coated with anti-red cell antibody of the IgM type and mouse serum as the source of complement. The percentage of cells binding these complexes is determined under the microscope. The test may be performed on lymphoid cells which have been disassociated in a suspension or on frozen sections of lymphoid cells from spleen or lymph nodes. The EA receptor is detected in a similar fashion with red cells coated with anti-red cell antibody of the IgG type. The E binding human T cells can only be detected on lymphoid cells in suspension. Different red cell reagents can be coupled with either rhodamine or fluorescein. These reagents can then be used simultaneously to detect lymphoid cells bearing more than one receptor. Also the rosetted malignant cells can be placed on slides and stained, this procedure allows for definitive cytological identification.

Major Findings:

The cells from 8 patients with nodular lymphoma were studied, both in suspension and in section. The malignant cells of this lymphoma bound 19SEAC and are therefore B cells. In section, the 19SEAC reagent bound only over the cells in the malignant nodules. The cells in suspension

from three patients with poorly differentiated lymphoma (diffuse), bound E indicating that these cells are T cells. The cells from three patients with histocytic medullary reticulosis (Hairy cell leukemia) bound 7SEA indicating that these cells were monocytes.

Significance to Biomedical Research and the Program of the Institute:

Malignant lymphoid reticular cells preserve many of the receptors found on normal cells. The identification of these receptors on malignant cells thus serves to identify the origin of the malignant cells. The classification of malignant lymphons based upon their normal cell of origin may have to be useful both for diagnosis and prognosis. The availability of large numbers of uniform cells populations may provide a reagent for further study of the chemical nature of these receptors. The study of the products and physiologic functions of these malignant cells (see below) may provide clues as to the pathophysiology of certain amlignant disease states. Finally the presence of these descrete receptors on the surface of malignant cells may allow for a method of attacking and destroying these malignant cells.

Proposed Course of the Project:

The studies of human malignant lymphomas are being continued and expanded.

Project Subtitle II: Identification and functional study of the abnormal cells of the Sezary Syndrome and mycosis fungoides

Project Description:

Objectives:

The Sezary Syndrome and a related disorder mycosis fungoides are a type of lymphoma characterized by either diffuse or nodular involvement of the skin by neoplastic lymphoid cells. These cells may also appear in the peripheral blood. Late in the course of the disease, other organ systems may be come infiltrated and destroyed. Our objective was to examine the origin of the malignant cells involved in this disease and study some of their physiological properties.

Methods Employed:

The same methods used in the preceeding section were used to identify the receptors of these malignant cells. In addition functional characteristics of these cells were investigated; the properties studied included the production by these cells of migration inhibition factor (MIF) and the ability of these cells to act as either stimulator or responder cells in the mixed lymphocyte reaction.

Major Findings:

The abnormal lymphocytes from three patients with mycosis fungoides and four patients with lymphocytic leukemia and erythroderma were found to have properties of T cells (they formed rosettes with ummodified sheep cells (E) and lacked surface immunoglobulin). The cells of one of these patients, in addition to binding E, also bound 19SEAC and therefore had the C3 receptor characteristic of B cells.

Cells from one patients with the Sezary Syndrome were stimulated with PHA and could respond in mixed lymphocyte culture. The cells of two other patients with the Sezary Syndrome did not respond in vitro to PHA stimulation and did not respond in mixed lymphocyte culture. Moreover the cells from one patient failed to act as stimulator cells in mixed lymphocyte culture.

Sera from six patients with Sezary Syndrome were found to contain MIF like activity while sera from normal individuals and from patients wth other dermatologic disease did not contain MIF activity. Furthermore supernatants from cultures of the lymphocytes of 3 of 5 patients with the Sezary Syndrome contained MIF activity whereas supernatants of lymphocytes from normal individuals did not have MIF activity.

Significance to Biomedical Research and Program of the Institute:

The identification of the origin of the malignant cells of mycosis fungoides and the Sezary Syndrome and the functional abilities of those cells may provide important clues as to the pathophysiology of the disease process. For example unregulated production of lymphokines could conceivably be related to the severe erythroderma observed in this group of patients. Furthermore, the failure in some instances of these cells to stimulate mixed lymphocyte culture may provide important reagants for the study of the nature of the antigens on the surfaces of normal lymphocytes which are required for this in vitro immunological reaction. Finally, a better understanding of the physiology of these cells may provide a basis for more effective therapy of these diseases. More specifically knowledge of the long survival time and slow mitotic rate of the abnormal T cells present in Sezary snydrome and mycosis fungoides suggested that leukaphersis might be a useful form of therapy. In one patient, such therapy has indeed proven to be highly successful.

Project Subtitle III: Rapid induction of B cell tumors by Abelson virus

Objectives:

A recently described virus, the Abelson virus, is able to induce lymphomas in a variety of strains of mice with a very high frequency and

with a very short latent period (2-3 weeks). The objective of the present study was to determine the lymphoid cell type of origin of these malignant cells.

Methods Employed:

BALB/c mice were injected with the Abelson virus intraperitoneally. Mice were observed for tumors; the primary tumors were examined by the methods to be described below and were also transplanted subcutaneously in other BALB/c mice. The secondary tumors were also examined.

The cells were teased from the tumors and individual cell suspensions were prepared. These were labelled with ^{51}Cr and treated with anti θ serum and complement. The cells were also stained in the living state with fluorescein labelled anti κ serum or class specific antibody.

Major Findings:

No tumor cells were killed with anti-θ serum and complement. These results indicated that cells were θ negative and were therefore not likely to be malignant thymus-derived lymphocytes.

The cells prepared from primary tumors did not have immunoglobulin on their surface. However, the cells from secondary tumors had kappa chains for their surface as judged by the results of immunofluorescent staining. Of great interest was the fact that the cells did not stain with any class specific antibody tested. This result suggests that only free kappa chains are present on the surface of these malignant B cells.

Significance to Biomedical Research and the Program of the Institute:

Study of the basic biology of tumors induced by this new virus may give insights into the mechanism of viral oncogenesis. The apparent specificity of this virus for B lymphocytes raises the possibility that B lymphocytes may have surface receptors for this virus and therefore may be especially susceptible. In this regard, it is of interest that chronic lymphatic leukemia of man is principally a disease of B lymphocytes and that a receptor for the EB virus is also found only on B lymphocytes.

Proposed Course of the Project:

Further studies of the host range of this virus will be conducted - specifically if the Abelson virus or one of its varients can produce tumors in inbred guinea pigs.

Publications:

Shevach, E.M., Jaffe, E.S., Green, I.: Receptors for complement and immunoglobulin on human and animal lymphoid cells. Transplantation Reviews 16: 3, 1973.

Chusid, T.M., Hardin, J., Frank, M.M. and Green, I.: Identification of Cells infiltrating the minor salivary glands in patients with Sjögrens syndrome. J. Immunol. 112: 641, 1974.

Shevach, E.M., Edelson, R.L., Frank, M.M. and Green, I.: A human leukemia cell with both B and T cell surface receptors. Proc. Nat. Acad. Sci. 71: 863, 1974.

Jaffe, E.S., Shevach, E.M., Frank, M.M., Berard, C.W., Green, I.: Nodular lymphoma: Evidence for origin from follicular B lymphocytes. New England J. Med. 290: 813, 1974

Jaffe, E.S., Shevach, E. M., Frank, M.M. and Green, I.: Leukemic reticuloendotheliosis: Presence of the histiocyte IgG receptor. Am. J. Med. "In press".

van Boxel, J.A., Hardin, J.A., Green, I. and Paul, W.E. Multiple heavy-chain determinants on individual B lymphocytes in the peripheral blood of patients with Sjögrens syndrome. New England J. Med. 289: 823, 1974.

Jaffe, E.S., Shevach, E. M., Sussman, B.S., Frank, M.M., Green, I. and Berard, C.W. Membrane receptor sites for the identification of lymphoreticular cells in benign and malignant conditions. Brit. J. Cancer. "In press".

Stobo, J., Green, I., Jackson, L. and Baron, S. Identification of a subpopulation of mouse lymphoid cells required for interferon production following stimulation with mitogens. J. Immunol. 112: 1589, 1974.

Green, I., Jaffe, E.S., Shevach, E.M., Edelson, R.L., Frank, M.M. and Berard, C.W. Determination of the origin of malignant reticular cells by the use of surface membrane markers. Monograph - Int. Acad. Path. "In press". 1974.

van Boxel, J.A., Paul, W.E., Green, I. and Frand, M.M. Antibody-dependant lymphoid cell-mediated cytotoxicity:Role of complement. J. of Immun. 112:398, 1974.

Serial No. NIAID - 144
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Differentiation and Activation of Mouse T and B Lymphocytes

Previous Serial Number: None

Principal Investigator: Dr. Donald E. Mosier

Other Investigators: Project Subtitle I: Dr. W.E. Paul, LI/NIAID
Dr. R. Asofsky, LMI/NIAID
Dr. R. Tigelaar, LMI/NIAID
Dr. P.L. Cohen, LI/NIAID
Dr. I. Scher, Naval Medical Research Res. Institute
Dr. P.R.B. McMaster, LMI/NIAID

Project Subtitle II: Dr. P.L. Cohen, LI/NIAID
Dr. S. Cross, Micro. Assoc.
Dr. H.C. Morse, III, LMI/NIAID

Cooperating Units: LMI/NIAID
Naval Medical Research Institute
National Naval Medical Center, Bethesda, Maryland
Microbiological Associates, Bethesda, Maryland

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to	Guest Workers	TOTAL
Total	3			3
Professional	2			2
Other	1			1

Project Subtitle I: Induction and regulation of immune responses in vitro

Project Description:

Objectives:

The immune responses to some antigens requires little or no cooperation from thymus-derived (T) lymphocytes or adherent accessory (A) cells. These responses to a defined set of antigens constitute a

unique model for studing direct activation of antibody-forming bone marrow-derived (B) lymphocytes. The objective of this work is to understand how the interaction of antigen with the surface of a B lymphocyte leads to the induction of specific antibody synthesis.

Methods Employed:

Mouse spleen cells are cultured in vitro with antigen for 4 or 5 days and the number of cells releasing specific antibody of the IgM or IgG class enumerated by localized hemolysis of antigen-coated erythrocytes in agarose.

Major Findings:

Mouse spleen cells were found to respond to dinitrophenyl-lysine coupled to polysucrose (DNP-Ficoll, prepared by Dr. P.R.B. McMaster, LMI/NIAID) by producing large numbers of IgM antibody-forming cells in vitro. This response to a novel antigen has been studied extensively. It requires neither T lymphocytes nor macrophage-rich adherent cells for the generation of antibody-forming cells. The magnitude of the response is unaffected by priming spleen cell donors with DNP-protein antigens. Neither Ficoll nor DNP-Ficoll are significantly mitogenic, nor do they bind or activate complement components. Induction of DNP-specific tolerance abrogates responsiveness to DNP-Ficoll, showing a requirement for DNP-specific cells to initiate a response. Large amounts of Ficoll added to cultures simultaneously with a small immunogenic quantity of DNP-Ficoll fail to alter the anti-DNP response, suggesting that the responding B lymphocyte recognizes and is stimulated primarily via DNP haptenic groups. In experiments done in collaboration with Dr. R. Asofsky (LMI/NIAID), goat anti-μ serum was found to be a potent inhibitor of the DNP-Ficoll response, suggesting immunoglobulin as the cell receptor for DNP.

In the course of tolerance experiments using the DNP derivative of a copolymer of D glutamic acid and D lysine (DNP-D-GL) to inhibit the response to DNP-Ficoll, we discovered that DNP-D-GL itself is a good in vitro immunogen under a circumscribed set of conditions. Namely, DNP-D-GL bound to adherent cells from mouse spleen, or presented free in exceedingly low doses (in the picogram range) stimulates a significant anti-DNP primary in vitro response. However, DNP-D-GL presented in higher concentrations or in the absence of adherent cells seems to lead to irreversible DNP-specific tolerance. This system is also without a requirement for T lymphocytes, and is being used to study immunogenic and tolerogenic signals in vitro.

In collaboration with Dr. R. Tigelaar, an in vitro response to type III pneumococcal polysaccharide (SIII) has been induced. These experiments are in a preliminary phase, but they may well lead to a system for defining T cell regulation of antibody synthesis in vitro, and confirming and extending the observations of Dr. P. Baker et. al. on T suppressor cells and the SIII response.

In collaboration with Dr. Philip Cohen and Dr. Irwin Scher, the response of genetically B cell deficient CBA/HN mice is being studied. Spleen cells from such mice fail to respond to DNP-Ficoll in vitro, but respond well to sheep erythrocytes, a T-dependent antigen. The nature of the B cell defect in these mice is being examined in cell reconstitution experiments in vitro.

Significance to Biomedical Research and to the Program of the Institute:

The use of antigens capable of direct B cell activation or inactivation will allow an understanding of regulation of immune responses at the cellular level. In the cases of DNP-Ficoll and SIII, these polysaccharide antigens are appropriate models for human immune responses to bacterial cell wall polysaccharide antigens.

Proposed Course of the Project:

A minimal model for antibody induction in vitro will be studied, using purified B lymphocytes stimulated with DNP-Ficoll, to further delineate the events involved in cell activation. Analysis of B cell subpopulations will be carried out using the CBA/HN strain.

Project Subtitle II: Ontogeny of T lymphocyte function.

Project Description:

Objectives:

Thymic-derived (T) lymphocytes mediate cellular immunity and appear to regulate many functions of bone marrow-derived (B) lymphocytes. The differentiation of T lymphocytes in the developing animal is thus of clear interest; moreover, subpopulations of T cells may manifest their function at different times in ontogeny, and therefore provide insight into their role in the adult, where several T cell subpopulations seem to coexist.

Methods Employed:

Thymocytes or spleen cells from fetal, neonatal, or adult BALB/c mice have been cultured in vitro with a variety of specific and nonspecific stimuli of T and B lymphocytes and proliferation or antibody formation assayed. Steroid pretreatment or infection with thymic agent, a herpes virus, have been used to selectively alter T cell function.

Dr. Philip Cohen and I have continued to study to acquisition of T lymphocyte function in the neonatal BALB/c mouse. Two model systems are being used to selectively alter the proportions of T cell subpopulations in the neonate. Hydrocortisone administration has been found to reduce the number of lymphocytes in the neonatal thymus by 80-90%, but to increase the responsiveness of the surviving subpopulation to phytomitogen stimulation by six- to eight-fold, establishing the existence of a

highly-reactive subpopulation of steroid-resistant cells in the newborn as well as in the adult. In the second system, Drs. P. Cohen, S. Cross, H.C. Morse, III, and I have been studying the effects of neonatal infection with the herpes virus, thymic agent (TA), which induces reversible thymic necrosis when inoculated soon after birth. TA-infected BALB/c mice show severe depression of T-lymphocyte mediated responses in both thymus and spleen for the first 5 or 6 weeks of life. B lymphocyte responses, such as proliferation after lipopolysaccharide stimulation or antibody formation to DNP-Ficoll, are unimpaired. The recovery phase of this transient T cell defect is now being examined in greater detail.

The ability of T lymphoyctes to act as "helper" cells for antibody formation during ontogeny has been examined by comparing the ability of neonatal BALB/c spleen cells to form antibody in vitro in response to the T-dependent antigen sheep red blood cells (SRBC) or the T-independent antigen DNP-Ficoll. The ability to respond to DNP-Ficoll appears prior to the ability to respond to SRBC, suggesting a lag in development of T helper cells until 3 or 4 weeks of age. This interpretation was confounded, however, by the finding that T cells (purified by nylon wool columns) from neonatal spleen were suppressive of antibody formation. Apparently the neonatal spleen has both a deficit of T helper cells and an excess of T suppressor cells.

In collaboration with Dr. H.C. Morse III, the ability of a long-term cell line derived from thymic epithelium (TEP's) to reconstitute the T cell defect of congenitally athymic nude mice has been examined. In control experiments, TEP's were found to fully reconstitute all T cell functions measured in neonatally-thymectomized BALB/c mice. In contrast, nu/nu athymic (BALB/c) mice, which have been stated to have an epithelial cell defect, were resistant to repeated attempts at reconstitution with TEP's. Either nude mice have a stem cell defect, or neonatally-thymectomized mice already have differentiated "prethymic" T cells susceptible to epithelial influence prior to birth.

Significance to Biomedical Research and the Program of the Institute:

Understanding the ontogeny of T cell function is crucial to understanding a variety of immunological defects in the newborn human, e.g. increased susceptibility to viral infection or inability to reject allogeneic cells acquired during blood transfusions. It may also lead to a fuller understanding of self-tolerance.

Proposed Course of the Project:

The work on thymic agent and the mechanism of inductive influence by thymic epithelial cells will be expanded. The role of neonatal T suppressor cells in inducing self-tolerance will be tested using cell fractionation on gradients and transfer to T-depleted recipients. The role of viral infection in altering T cell function will be the subject of several new studies.

Publications:

Mosier, D.E.: Ontogeny of mouse lymphocyte function. I. Paradoxical elevation of reactivity to allogeneic cells and phytohemagglutinin in BALB/c fetal thymocytes. J. Immunol. 112:305, 1974.

Mosier, D.E., Johnson, B.M., Paul, W.E., and McMaster, P.R.B.: Cellular requirements for the primary in vitro antibody response to DNP-Ficoll. J. Exp. Med. In press (May , 1974).

Mosier, D.E. and Cohen, P.L.: Ontogeny of mouse T lymphocyte function. Fed. Proc. (Symposium presented at 58th Annual FASEB meeting, April, 1974). In press.

Serial No. NIAID - 145
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Specificity in Immune Responses

Previous Serial No.: Same

Principal Investigator: Dr. John K. Inman

Other Investigators:

Project Subtitle	I:	Dr. David Alling (NIAID)
		Dr. Bruce Merchant (BOB, FDA)
		Dr. Ramesh Srivastava (BOB,FDA)
		Dr. Ettore Appella (NCI)
Project Subtitle	II:	Dr. Bruce Merchant (BOB, FDA)
		Dr. Ettore Appella (NCI)
		Dr. Hillel Koren (NCI)
Project Subtitle	III:	Dr. Bruce Merchant (BOB, FDA)

Cooperating Units: Bureau of Biologics, FDA (Drs. Merchant and Srivastava), Laboratory of Cell Biology, National Cancer Institute (Dr. Appella), Immunology Branch, National Cancer Institute (Dr. Koren).

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	2 9/12			2 9/12
Professional	1 9/12			1 9/12
Other	1			1

Project Subtitle I: Multispecificity of Antibodies

Project Description:

Objectives:

The overall objectives of this project are to relate immunological specificity to the properties of individual antibody and antibody-like molecules (receptors), and to probe the extent and genetic basis of antibody diversity. Specific objectives are: (1) to provide a quantitative theoretical framework for understanding immunological specificity in terms of multispecific properties of antibody combining sites, (2) to explore experimentally the extent and character of disparate-structure multispecificity, (3) to employ disparate-structure cross reactions as a means for raising relatively homogeneous antibody for amino acid sequence

studies, (4) to use such cross reactions also as markers for variable region immunoglobulin genes, (5) to develop improved methodology for amino acid sequencing of peptides and proteins, and (6) to sequence variable regions of antibodies isolated by immunoadsorption based on multiple binding specificities.

Methods Employed:

Hapten-specific, hemolytic plaque assays have been carried out by methods developed over a number of years in Dr. Merchant's laboratory. These procedures have included storage of cell suspensions for long periods of time under liquid nitrogen and their restoration to a viable state. The studies of humoral immune specificity using these assays have been greatly enhanced by the use of a panel of 14 to 16 large haptens and a gentle, systematic coupling method (Inman et al., Immunochem. 10, 153,165 (1973).

Polyacrylamide adsorbents are being prepared by chemical procedures developed by Inman and Dintzis (Biochem. 8, 4074 (1969) and recently extended by Inman (see publications). Immunoglobulins (7S) from normal mouse sera were prepared by precipitation with 50% saturated ammonium sulfate followed by dialysis and column chromatography on DEAE-cellulose. All compounds used in studying new radioiodination and sequencing procedures were synthesized in this laboratory.

Major Findings:

Recent evidence indicates that individual antibody molecules can bind substances with structures quite different from that of the eliciting antigen. These disparate-structure cross reactions contrast with the exquisite specificity commonly associated with serological reactions. In the latter case, however, one is dealing with populations containing many molecular species of antibody. The member species of each population are structurally diverse but share a common reactivity to one or more determinant structures of the immunogen whereas disparate reactivities may be largely unshared.

Work has been completed on a theoretical model which provides a means for calculating the probability of serological cross reactions within a system of multispecific antibodies as described above. Combinatorial logic was applied to arrive at a general probability function involving 4 independent variables. These parameters are: the total number of combining species of antibody, the portion thereof that can potentially react with a test hapten, the number of species in an antiserum (made to another hapten), and the number of antiserum species that cross-react with the test hapten. A computer program was set up by Dr. David Alling to evaluate probabilities for any number of cross-reacting species covering wide ranges of the 4 variables. Approximation formulas were derived which allowed interpolations to be made by manual calculation. Several results were quite noteworthy: if

one assumes a reasonable, finite threshold for detecting serological cross reactions, then it could be shown that (1) the specificity of immune sera can exceed by many orders of magnitude that of its individual components, (2) the specificity of immune sera increases sharply (and exponentially) with increasing degree of heterogeneity, and (3) adequately specific immune sera to an unlimited number of antigens can be populated from a total repertoire of a few hundred thousand antibody species. If nature has taken this conservative course, then the genetic load for coding the corresponding variable region structures would be rather modest even if all the information were carried in the germ line. A more detailed account of the model and the above results will be published later this year (see publications). A conceptual model of a multispecific binding region on an antibody surface was proposed where binding is viewed as a selection of a dozen or so surface atoms for short-range interactions with the antigen (from a much larger set of potential contact atoms). This idea has been far more useful in dealing with structure-specificity relationships than the conventional complementary surface (lock and key) concept.

Studies with the hapten-specific hemolytic plaque assay in collaboration with Dr. Bruce Merchant have continued to reveal numerous instances of multispecific binding by anti-hapten antibodies. Some antibody secreting cells from most animals immunized with substituted phenylazo haptens formed plaques with red cells coupled with haptens of dissimilar structure such as tripeptide derivatives of DNP, TNP, dansyl, and more recently NP and NIP (3-nitro-4-hydroxyphenylacetyl and 3-nitro-4-hydroxy-5-iodophenylacetyl, respectively).

A more direct study of disparate-structure cross reactions has been carried out in collaboration with Drs. Merchant and Srivastava. Cross reactions were detected by inhibition of plaques caused by various compounds (unrelated to the immunogen) placed in the assay medium. Since disparate-structure cross-reactions involve only one or a few member species of a heterogeneous population of antibodies, inhibition of plaques by such means would seldom cause a significant percentage decrease in the PFC. However, measurable plaque inhibition can be seen if restricted populations of clones are tested. This has been accomplished either by using spleen cells from a restricted responder animal or by employing test red cells sensitized with an alternate hapten. Thus, in the latter case, plaque inhibition demonstrates a triple specificity (to the immunogen, the red cell hapten and the soluble test hapten). A panel of 20 diverse test haptens has been employed. With most immune spleen cell suspensions tested, at least one and often several test compounds (at 10^{-4} and 10^{-5} M) inhibited greater than 30% of the PFC directed against an alternate hapten. All 20 compounds were found to be non-inhibitory with one or more cell donors. Therefore, inhibition by toxic suppression of antibody synthesis can be ruled out. Cells from three rabbits, immunized with a p-sulfophenylazo coupled carrier, were tested against erythrocytes bearing the trimethylphenylammonium hapten. Results showed PFC inhibition with 2 out of the 20 test compounds.

Spleen cells from a rabbit giving a highly restricted secondary (IgG) response to the p-arsonophenylazo (A) hapten were tested. Restriction was assessed by isoelectric focusing (by Dr. Mage) and with PFC inhibition curves with free, homologous hapten. Two of the 20 compounds caused substantial inhibition (approx. 50-55%) of PFC against the homologous hapten. A littermate and three other NZW rabbits gave non-restricted responses to A by isoelectric focusing and showed no PFC inhibition by any of the 20 compounds. The results support the view that disparate-structure cross-reactions are detectable when one deals with highly restricted populations of antibodies. Myeloma proteins, which behave in a similar manner, appear then to be functional representatives of normal antibody species.

Currently a more general approach to studying disparate-structure cross-reactions is being initiated in my laboratory. Rather than observing a secondary phenomenon such as specific hemolytic lysis, measurements will be made of the direct interaction of specific antibodies with various haptens bound to immunoadsorbents. A panel of such immunoadsorbents is currently being prepared. The selected adsorbent matrix is cross-linked polyacrylamide. Haptens are being attached to the polymer backbone through spacing groups. During the past year a series of versatile spacing structures bearing terminal functional groups were designed and their synthesis worked out. Details of these procedures are being published in a forthcoming volume of "Methods in Enzymology" devoted to methods for affinity chromatography (see publications). The spaced-out terminal groups are useful in forming stable covalent bonds with a large variety of haptens. The groups include α and β primary amines, carboxyls, carboxylic hydrazides, α halogen, arylisothiocyanate, and sulfhydryls.

Specific antibodies being screened for cross-reactivity are to be labeled with radioiodine. In the interest of minimizing chemical alteration (and possibly binding properties) of antibodies by the radioiodination procedure, new labeling methods have been investigated which eliminate exposure of the test protein to oxidizing or reducing substances and preclude the involvement of tyrosine residues. The favored approach has been the attachment of small iodine-containing compounds to free amino groups of immunoglobulin molecules by means of a mild, specific acylation reaction. Currently, immunoglobulins are being labeled by a modification of the Bolton and Hunter procedure (Biochem. J. 133, 529 (1973)). The reactive N-hydroxysuccinimide ester of p-hydroxyphenylpropionic acid is iodinated by the chloramine-T method and then extracted into benzene. After removal of solvent the iodinated compound is dissolved in water and added to a buffered solution of the protein to be labeled. Very useful modifications of the above procedure have been developed recently in this laboratory.

A similar approach to radioiodination has been under investigation in collaboration with Dr. Srivastava. Radioiodide can be exchanged into active esters of iodoalkanoic acids in acetone solution. We have investigated a number of such compounds with regard to rates of exchange and stability of the label. The most satisfactory results were obtained with the N-hydroxysuccinimide ester of 6-iodohexanoic acid. These esters and N-acylsuccinimides of various C_3 through C_6 iodoacids were compared.

The sensitivity of the screening assay was established by measuring the natural antibody level to the NIP hapten in the 7S immunoglobulins of unimmunized SM/J and CBA/H mice. The labeled immunoglobulin was passed through a small NIP-conjugated, polyacrylamide bead column. The column was washed with buffer and eluted with the free hapten (at 5×10^{-4} M). A distinct peak was obtained representing only 0.09% of the total radioactivity of the sample.

Some progress has been made in developing new methodology in amino acid sequencing. Although automated sequential degradations have greatly accelerated this type of analysis, many technical problems remain, especially in the area of identification and quantitation of degradation products. I have found that thiazolinones, which are liberated upon stepwise degradation of peptides, can be rapidly converted to phenylthiocarbamyl amino acid alkylamides by treatment with volatile alkylamines. These derivatives were synthesized by several routes and were found to be separable by TLC and liquid chromatography. Their ultraviolet spectra lend well to high-sensitivity, on-stream UV detection.

Significance to Biomedical Research and the Program of the Institute:

It is now possible to develop and use methods of high sensitivity and discriminatory power for exploring more fully the degree and nature of antibody specificity. It has become evident that the specificity of immune sera, and presumably of complex immune systems, may not be a direct reflection of the specificity of individual combining sites on antibodies or receptors. An understanding of the specificity of immune systems will have to rest on a fundamental reassessment of binding site-ligand specificity and on finding the principles which relate this to specificity shown by more complex systems. These principles will involve effects of homogeneous and heterogeneous site populations and various kinds of polyvalent (polysite) ligation. These findings should help to clarify many questions concerning the operation of control mechanisms in humoral and cell-mediated immunity.

Fundamental questions about the genetic and biosynthetic mechanisms operating to generate antibody diversity remain unanswered. It is difficult to believe that the processes which operate for this purpose are an entirely unique adaptation. Most investigators feel that solutions to problems posed by antibody diversity will help in answering many important questions in molecular and cell biology of higher organisms.

Proposed Course of Project:

Theoretical problems will lie in the area of polyvalent ligation and its effects on specificity. A body of data from hapten-specific plaque assays will furnish support for ideas which have already taken shape. The screening program for disparate-structure cross-reactions will have immediate priority over other concerns. Once established the screening will be used for setting up cross stimulation experiments for producing restricted antibody. Cross-reactions will also be used in establishing markers for V-region genes. Three-way cross-reactions may prove effective. The markers will be employed

to find out how frequently a V_L x V_H structure will occur in (at least some) progeny of an inbred animal in order to establish the extent of germ line transmission (or somatic generation) of diversity.

V-regions markers will be checked where possible by amino acid sequencing of V-regions of H and L chains or portions thereof. New automated systems (based on the solid-phase principle) for sequencing will be explored in the near future in collaboration with Dr. Appella. The analytical methods discussed above will be employed for identification of various amino acids.

Project Subtitle II: Manipulation of the Immune Response: Synthesis and/or Chemical Modification of Antigens.

Project Description:

Objectives:

To employ various strategies of chemical synthesis in the design and preparation of antigenic reagents which are uniquely suited for use in answering questions about genetic and receptor-mediated control in immune responses. These efforts are carried out in collaboration with other investigators who specialize in techniques of immunobiology. In most instances there is a special demand for chemical steps that can be carried out in aqueous media and, in some cases, under very mild conditions in the presence of living cells.

Methods Employed:

Most reactions, except for the synthesis of intermediates, are conducted in aqueous solutions. Many of the mild acylation reactions used in peptide synthesis have been adapted for these studies: Thus, frequent use has been made of acyl azides and active esters. Removal of reagents from reactions involving soluble polymers has been performed by dialysis. Alkylation reactions involving sulfhydryl groups are currently being investigated as a mild, specific bond-forming method.

Major Findings:

A general and very useful approach to introducing haptenic groups on the thymus independent carrier, Ficoll (polymerized sucrose from Pharmacia), has been developed. Carboxymethylation (forming stable ether bonds at sucrose OH groups) is carried out to a controlled degree by a time-limited reaction with chloroacetate in the presence of 1 to 2 M sodium hydroxide at room temperature (or at 40°). Haptens may be subsequently attached to the bound carboxyl groups. So far, the latter have been altered to provide primary amino groups by reaction with water-soluble carbodiimide in the presence of excess ethylenediamine dihydrochloride. Haptens have then been bound to the terminal amino groups. Several of the large tripeptide derivatives (see use in Project Subtitle III), DNP, and TNP groups have been coupled to Ficoll by this route.

Another general strategy of considerable potential use is the heteroligation of two different proteins (or polymers), that is, the predominant formation of A-B (rather than A-A or B-B) covalent complexes. Very few, if any, useful techniques exist for this purpose. Two new reagents have been designed and synthesized during the past year for performing heteroligation involving proteins and viable cells. One is a compound for introducing sulfhydryl groups onto proteins or cell surfaces under very mild conditions (at physiological pH without the requirement for catalysts or blocking groups). The other reagent binds mild alkylating groups to proteins or polymers containing free amino groups. Two components, each treated with one or the other reagent, can be made to combine selectively through a stable thioether bond. This approach is now being evaluated.

Studies of specificity and mechanisms in cell-mediated immunity employing complete in vitro systems are being carried out by Dr. Hillel Koren with my collaboration on chemical problems. Both sensitizing and target cells are prepared hapten-specific by conjugation with the tripeptide derivatives of Inman et al. (as described in Immunochem 10, 153,165 (1973)). Viability and function have been maintained in spite of heavy hapten loading of cell surface components.

Significance to Biomedical Research and the Program of the Institute:

Chemically synthesized or modified antigens are playing an important part in numerous studies aimed at elucidating mechanisms of immune responses. Rational design and control of antigenic structure permits separate evaluation of effects due to carrier and hapten specificity, genetic control of responsiveness, various routes of antigen processing, and modes of presentation of antigen to precursor cells. From these basic studies will evolve ways of manipulating immune responses through use of specially designed antigens. These findings will thus set the stage for rational approaches to immunotherapy and to the treatment of diseases of immunological origin.

Proposed Course of Project:

The colloborative studies described above are to continue. Emphasis will be placed on the preparation and use of thymus independent antigens (e.g., those based on Ficoll) and on protein-cell conjugates. The latter will be used in experiments aimed at enhancing cell-mediated immunity and will be a collaborative effort with Drs. Appella and Law (NCI). Plans are currently under consideration for antigen modifications useful in exploring the specificity of immune responsiveness control and introducing radioactive labels of high specific activity into synthetic antigens.

Project Subtitle III: Cellular Studies on the Development of Specificity in Humoral Immune Responses: B cell Specificity to Thymus Independent Antigens

Project Description:

Objectives:

1. To determine whether immune responses to thymus independent antigens are as diverse with regard to specificity as they are in response to thymus dependent antigens.

2. To determine whether pre-existing, spontaneous, background plaque-forming cell activity is as diverse in athymic mice as are corresponding activities in intact mice.

3. To determine optimal levels of hapten derivatization and dose for thymus independent antigens.

4. To ascertain whether immunization with a thymus independent antigen will activate precursor T cells in athymic or T cell deficient animals.

Methods Employed:

Athymic (nude) mice were obtained, quarantined, and produced in the specific pathogen free area of the NIH animal production unit. The athymic mice and normal heterozygous controls produced on an outbred NIH Swiss albino background were utilized for studies of plaque-forming cell production following immunization with hapten derivatized aminoethylcarbamylmethyl-(AECM)-Ficoll. In some experiments athymic and normal mice were compared in their ability to respond to haptenated Ficoll or to thymus dependent antigens such as burro red cells. Following immunization with any given haptenated Ficoll, PFC assays were regularly accomplished with erythrocytes derivatized with the immunizing hapten and with other erythrocytes conjugated with a panel of structurally related haptens.

Major Findings:

The studies reported below were carried out largely in the laboratory of Dr. Bruce Merchant as a collaborative project.

Spontaneous background, hapten-specific plaque activity in athymic (nude) mice is regularly comparable to pre-existing, spontaneous activity in normal heterozygous controls. This indicates that the propensity to activation of precursor B cells in nude mice is comparable to that in normal mice of similar genetic background.

Previous studies had demonstrated that multiple populations of hapten-specific plaque-forming cells arise in response to immunization with a single chemically defined haptenic determinant. In most instances, the vast majority of such "quasi-specific" plaque-forming cells are directed against structural analogs of the immunizing hapten. It was unclear whether this diversity in the specificity of B cell responses was attributable directly to an intrinsic characteristic of B cells, or whether it could be due to some extraneous effect of T cells imposed upon B cells at the time of their cooperative interaction in response to thymus dependent antigens. Employment of athymic mice

allowed elucidation of this question. Athymic mice produced diverse immune responses composed of multiple coexisting populations of hapten-specific plaque-forming cells. The extent of diversity and the magnitude of responses observed in these animals was very comparable to that previously observed in intact normal mice from a number of inbred strains. Furthermore, the diversity of specific plaque-forming cell responses found in athymic mice was nearly the same as that observed simultaneously in normal, heterozygous controls.

Both athymic and heterozygous mice were immunized with p-azobenzenearsonate-TGG-(A-), p-azobenzenesulfonate-TGG-(S-), and p-azophenyl-TGG-(ϕ-) conjugated AECM-Ficoll at both high and low levels of derivatization. In all instances, the more highly conjugated preparations were more immunogenic. Nearly equivalent immune responses were obtained with A_{45}-Ficoll, S_{61}-Ficoll and ϕ_{76}-Ficoll. In no instances were typical secondary immune responses observed following second injection of any of these antigens. Dose response studies demonstrated that immune responsiveness was obtainable between 10 nanograms and 100 micrograms. Optimal responses were normally obtained with about 10 micrograms of antigen. Even under optimal immunizing conditions no typical secondary plaque-forming cells were seen in response to S_{61}- or A_{45}-Ficoll in any of 6 inbred strains of mice. Resurgence of IgM-secreting cells was regularly observed. However, such secondary response activity never equaled or exceeded the primary IgM response.

Significance to Biomedical Research and the Program of the Institute:

These studies indicate that haptenated Ficoll antigens are extremely useful tools for probing significant events that occur at the surface of precursor B cells and critical interactions that occur prior to their activation. Preliminary experiments indicate that they will be helpful in studying specific antibody-mediated suppression which abrogates the capacity of precursor B cells to respond to administered antigen. As more knowledge accumulates concerning their in vivo catabolism, this interesting class of haptenated immunogens may prove to be instrumental in dissecting significant interactions between macrophages, T cells and B cells.

Proposed Course of Project:

Many interesting leads have turned up in the work done so far. Further studies with haptenated Ficoll and possibly other thymus independent carriers will follow. Work is in progress on the question of whether thymus independent antigens act strictly in the absence of T cell function or whether they first induce an effective T cell population from T cell precursors known to be present even in athymic mice. Preliminary experiments suggest that the former alternative may be the case.

Honors and Awards:

Member of the Board of Reviewers of Federation Proceedings. Invited Speaker at the ICN-UCLA Symposium on Molecular Biology: The Immune System, March, 1974, Squaw Valley, California.

Publications:

Appella, E. and Inman, J.K.: The primary structure of rabbit and mouse immunoglobulin light chains: Structural correlates of allotypy. In: Contemporary Topics in Molecular Immunology, Vol. #2 Reisfeld, R.A. and Mandy, W.J. (Eds.) New York, N.Y., Plenum Publishing Corp., pp. 51-77, 1973.

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Serial No. NIAID - 146
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 - June 30, 1974

Project Title: Chemical Genetic and Immunochemical Studies of Immunoglobulins: Use of Genetic Markers in Studies of Lymphoid Differentiation and the Regulation of Gene Expression and Immune Responses

Previous Serial Number: 146

Principal Investigators: Rose G. Mage, Ph.D. and Glendowlyn O. Young-Cooper, M.S.

Other Investigators: Dr. A. Ansari, LI/NIAID
Dr. M. Carta, LI/NIAID
Dr. A. Drexler, LI/NIAID
Dr. S. Carta, LI/NIAID and LCB/NCI
Dr. E. Appella LCB/NCI
Dr. M. Waxdal, LI/NIAID

Cooperating Units: Dr. S. Dray, Chicago, Ill., Dr. R. Cathou, Boston, Mass., Dr. M. Freedman, Toronto, Canada, Dr. S. Tosi, Rome, Italy, Dr. A. Chersi, Rome, Italy

In addition to the above investigators, this year the following investigators have used rabbits of defined allotype which we have supplied and/or have received specific reagents which we have prepared: Dr. R. Asofsky, LMI/NIAID, Dr. T. Borsos, BGY/NCI, Dr. M. Bosma, Phila. Pa., P. Berman, Hanover, N.H., Dr. P. Baker, LMI/NIAID, Dr. D. Benjamin, Charlottesville, Va., Dr. J. Cebra, Baltimore, Md., Dr. M. Goldman, Chicago, Ill., R. Goodfleisch, Madison, Wisc., Dr. L.A. Herzenberg, Stanford, Calif., Drs. W.C. Hanly, and K. Knight, Chicago, Ill., Dr. J. Kimball, Medford, Ma., Dr. A. Lawton, Birmingham, Ala., Dr. W. Mandy, Austin, Tex., Dr. W.E. Paul, LI/NIAID, Dr. R. Reisfeld, LaJolla, Calif., Dr. B. Wolf, Phila., Penn., Dr. J. Wunderlich, I-NCI, Dr. T.J. Yoo, Iowa City, Iowa, Dr. M. Phillips, Walter Reed, Dr. P. Plotz, A&A/NIAMDD, Dr. M. Potter, LCBGY/NCI, Dr. C. Moroz, Peta Tikvah, Israel, Dr. E. Okazaki, Denver, Colo., Dr. S. Zappacosta, Naples, Italy. We have also typed serum samples for numerous investigators.

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned To Project	Guest Workers	[illegible]
Total	4-11/12		6/12	5-5/12
Professional	3-11/12		6/12	4-5/12
Other	1			1

Project Description:

Objectives:

To define by combined immunological and chemical approaches, the chemical nature of structural differences between genetically controlled polymorphic forms of immunoglobulins and between different classes, subclasses, types and subtypes of their polypeptide chains. To utilize this information about the location and chemical nature of the markers on various chains in performing and interpreting breeding studies. To utilize defined reagents for various chains and markers in affecting phenotypic expression of alleles or chain types. The overall objective is to understand the genetic mechanisms which lead to the synthesis of highly specific antibody molecules and the nature of the genes which influence and control immune responses.

Methods Employed:

Rabbits of known allotype are bred: to produce homozygous rabbits of all combinations at four or more genetic loci; to produce heterozygotes for use in specific experiments and as F1 progeny in linkage studies; to produce F2 and backcross progeny in linkage studies; to produce allotype-suppressed rabbits; and to study the genetics of the immune responses to pneumococcal vaccines, DNP-protein conjugates, and other antigens.

Sera of rabbits from the above categories are collected and typed by gel diffusion and hemagglutination techniques for allotypes. The sera are used as sources of IgG and light and heavy polypeptide chains for chemical sequence studies and for immunizations of rabbits and other animals.

Rabbits of defined allotype are immunized with IgG, light chains or other antigens such as lysozyme, pneumococcal vaccines, hapten-protein conjugates and (for idiotype studies) purified antibodies or IgG fractions and subfractions. Antisera are tested by qualitative and quantitative immunologic and electrophoretic techniques.

Allotypes, types, subtypes and idiotypes are quantitated by: radial immunodiffusion; hemagglutination inhibition; quantitative precipitation of iodine labeled proteins with specific antisera; binding of labeled proteins

to immunoabsorbents; inhibition of binding of the labeled antigens to immunoabsorbents. Immunoabsorbents are also prepared and used to purify antibodies, antigens and peptides of known specificity.

Cells from newborn and developing normal and allotype suppressed homozygous and heterozygous rabbits are labeled with iodinated or fluorescein-conjugated anti-allotype reagents. They are also cultured in vitro and assayed for incorporation of labeled amino acids or tritiated thymidine after exposure to a variety of materials including mitogens and anti-allotype reagents.

Purified light and heavy chains or fragments of these are obtained from IgG of normal sera or specifically purified antibodies of known allotype as well as from sera of allotype-suppressed rabbits. These are studied using a variety of methods for sequence determination including automated sequencing.

In order to carry out all the studies, we breed and maintain large numbers of rabbits in our own colonies. We produce and characterize large volumes of antisera including anti-allotype antisera made with a variety of donor and recipient combinations, anti-idiotype antisera, antibodies of restricted heterogeneity, and other anti-immunoglobulin reagents. We purify normal and antibody immunoglobulins from large volumes of rabbit sera for use as immunizing antigens, for structural studies, and for other studies described below.

Major Findings:

Peptides with a locus allotypic determinants have been prepared from antibodies of restricted heterogeneity of a1, a2 and a3 allotypes and purified using immunoabsorbents with purified anti-allotype antibodies coupled to them. Characterization of the a3 peptide from a heavy chain of limited heterogeneity is progressing. We have found that the active peptide may require a disulfide bridge since activity is lost upon reduction and alkylation. Its elution position from an agarose column in 5 M guanidine-HCl indicates a MW of about 6,000 and about 3,000 after reduction. The amino acid composition is consistent with two peptides of about 30 residues each, linked by a disulfide bridge. Techniques for quantitative assays of the active inhibitory peptides are currently being evaluated.

We are investigating in a similar manner, the structures on light polypeptide chains responsible for the characteristic serologically detectable b-locus antigenic determinants. Peptides obtained by tryptic digestion of citraconylated b4 light chains of highly limited heterogeneity inhibit the precipitation of b4 light chains with anti-b4 antiserum. A quantitative assay of inhibitory activity has been developed for measuring the inhibition of precipitation of ^{125}I labeled b4 light chains by anti-b4 antiserum. Preliminary results indicate that the peptides have high affinity for the antibody, and can inhibit light chains when mixed in approximately equimolar quantities. Since the antibodies are raised by immunization with whole IgG, we will be evaluating the conformation of b4 determinants on

free light chains, vs. IgG vs. smaller peptides. Immunoabsorbents with specifically purified anti-b4 antibodies coupled to them are being used for further purification and characterization of peptides with b4 determinants.

Light chains of lambda type were isolated from the sera of rabbits totally suppressed for expression of b5 kappa type by neonatal exposure to anti-b5 antisera. The light chains have considerable chemical homology with myeloma lambda chains of man and mouse. The five half-cysteine-containing peptides were isolated and shown to have amino acid sequence homologies with known structures of lambda chains in other species. A number of variants of the V-region peptide including cysteine 90 were found, and a partial sequence of one of these was determined. It was noted that the c7 and c21 allotypes are probably not due to amino acid substitutions in the cysteine peptides of the constant region, since no variants were found.

An anti-SIII antibody of highly restricted heterogeneity was isolated and found to give three closely spaced bands on isoelectric focusing. Surprisingly, these components could be separated preparatively by ion exchange chromatography on DEAE Sephadex. The light and heavy chains of each component were separated and amino acid analyses were done. The light chains appear to be identical, and the N-terminal amino acid sequence is indicative of homogeneity. The heavy chains, however, may have sequence differences. These are being evaluated by production of CNBr fragments of the heavy chains and structural studies of them. IgG and Fab fragments of each component have also been examined by circular dichroism. The CD spectra of the three components and of the Fab fragments of each component are indistinguishable.

Immunoglobulins in partially restricted or restricted antisera to type III or type VIII polysaccharides were fractionated by preparative isoelectric focusing at various pH ranges. A number of fractions with significantly different isoelectric points were obtained from each antiserum. After complete reduction and alkylation, the light and heavy polypeptide chains of the focused fractions were examined by disc electrophoresis and by isoelectric focusing. A number of fractions were obtained which yielded single light chains, often with different electrophoretic mobilities. The method was shown to provide fractions of very limited heterogeneity from a single antiserum. The expression of allelic forms of immunoglobulin during immunization of heterozygous rabbits with pneumococcal vaccines was monitored using anti-allotype antisera specific for different forms of kappa chains (b-locus markers). We also used the a-locus markers which are very likely associated with the variable portions of heavy chains, and idiotypic determinants associated with a restricted subpopulation of the anti-polysaccharide antibodies of an individual rabbit. Fractions obtained by preparative liquid isoelectric focusing were enriched for one a and/or b locus allotype. Enrichment of a specific idiotype from about 11% in the starting serum to as high as 65% in a focused fraction was also obtained.

In b^5b^5 rabbits recovering from b5 allotype suppression, there are relatively normal numbers of peripheral blood lymphocytes with endogenously synthesized b5 immunoglobulin on their membrane, but markedly and disproportionately low levels of circulating b5 immunoglobulin. The capacity of peripheral blood cells from these animals to transform into blast cells and synthesize DNA in response to anti-b5 antisera or pokeweed mitogen *in vitro* was markedly subnormal. The b5 bearing lymphocytes in rabbits recovering from allotype suppression may be unable to give rise to b5 secreting cells due to a functional block of the activation and/or maturation of these precursor cells.

Allotype suppression by maternal antibody can be reversed either by foster nursing or by intraperitoneal injections of serum with paternal allotype. The phenomenon occurs with allotypes of both the a and b loci. We have previously shown that in doubly heterozygous rabbits, the proportion of a-locus (V_H) allotype expressed dictates the proportion of genetically linked C_γ marker. If the proportion of paternal a-locus allotype is suppressed by exposure to antibody to the father's allotype in neonatal life, the genetically linked C_γ allotype is also suppressed. We have been conducting the reverse of this experiment to see if neonatal exposure to antibody to a constant region marker will suppress its expression, and if so whether the linked variable region marker will be affected. In the first three litters we have examined, it is clear that no chronic allotype suppression was established. We have developed methods for quantitating the C_γ markers A14 and A15 in whole sera, and have shown that the adult levels in animals exposed to maternal antibody to the paternal type, or repeatedly injected after birth, are indistinguishable from normally expressed levels. Two additional litter are now under study in which the mother was making antibody to two different C_γ markers (A11 and A15) simultaneously. We hoped that this might provide a more potent suppressive reagent. It appears that there may be some effect upon expression of the paternal type in these animals.

We have continued to conduct breeding experiments designed to detect recombination between linked markers for the V_H and C_γ regions of rabbit heavy chains, but have observed no additional recombinants. Additional studies of the single recombination event we have previously documented are in progress. Specifically, in collaboration with Drs. Hanly and Knight, the allotypes on two subclasses of rabbit IgA present in the family of the recombinant rabbit are being defined. This required production of new, previously undescribed reagents. We expect that we shall soon be able to distinguish the IgA from the maternal and paternal chromosomes which were passed to the recombinant animal, and by analysis of its progeny, determine whether or not the recombination also involved the establishment of a new linkage relationship between V_H and C_α genes.

Significance to Biomedical Research and the Program of the Institute:

These studies help to define the genetic mechanisms which lead to the synthesis of highly specific antibody molecules and the nature of the genes which influence and control immune responses. Allotypic markers are valuable for the study of cell surface receptors and the role they play in the differentiation of antibody producing cells. Our studies also help delineate the chemical basis for the specificity of protein antigens and antibodies, the nature of structural and regulatory genes for immunoglobulins, and the complexities of mammalian genetic systems. The breeding studies and cellular studies which can be done with rabbits provide information applicable to the homologous human immunoglobulin system. In addition, the rabbit allotypes are analogous in many respects to cellular isoantigens involved in transplantation, cell-cell recognition systems, tumor antigens and other cell surface markers. An understanding of the chemical nature of these antigenic types, the factors which control their genetic expression, and their biological roles is thus of broad fundamental interest.

Proposed Course of Project:

We are continuing most of the studies described. Recent emphasis has been on the chemical and genetic studies. We are re-initiating some cellular studies at the present time. In particular we are interested in further evaluation of the ontogeny of B lymphocytes in the rabbit, and studies of the mechanism of allotype-suppression utilizing in vitro culture methods.

Honors and Awards:

Instructor: "Immunogenetics" NIH tutorial Seminar. Member editorial boards of Immunochemistry and Federation Proceedings and Allergy and Immunology Study Section. Invited Lecturer: Society of General Physiologists Symposium "Cellular Selection and Regulation in the Immune Response", Woods Hole, Ma. FASEB Conference on "Biology of Development and Aging"-Symposium on "Gene Regulation in Differentiation and Development". Invited Workshop Chairman: Genetics and phenotypic expression of variable regions, Squaw Valley, Calif. Invited Discussant: Brooke Lodge, Mich., Conference on "Immunological Tolerance Mechanisms and Potential Therapeutic Applications."

Publications:

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Werblin, T.P., Kim, Y.T., Mage, R., Benacerraf, B. and Siskind, G.W.: The generation of antibody diversity. II. Genetic influence on the binding of anti-hapten antibody. Immunol. In press, 1973.

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Harrison, M.R. and Mage, R.G.: Allotype suppression in the rabbit. I. Ontogeny of cells bearing immunoglobulin of paternal allotype and the fate of these cells after treatment with anti-allotype antisera. J. Exp. Med. 138: 764-774, 1973.

Mage, R.G., Carta-Sorcini, M. and Appella, E.: Peptides with a locus allotypic determinants. Ann. Immunol. (Inst. Pasteur) 125C, 97-98, 1974.

Mage, R.G., Young-Cooper, G.O. and Harrison, M.R.: Rabbit allotypes--Tools for the examination of gene expression in lymphoid differentiation. In Edelman, G.M. Ed. Cellular Selection and Regulation in the Immune Response. Raven Press, N.Y., pp. 71-82, 1974.

Young-Cooper, G.O. and Mage, R.G.: Neutralization of Allotype Suppression in rabbits. Immunol. 26: 809-811, 1974.

Appella, E., Chersi, A., Rejnek, J., Reisfeld, R. and Mage, R.: Rabbit immunoglobulin lambda chains. Isolation and amino acid sequence of systeine-containing peptides. Immunochem. In press, 1974.

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Mage, R.G., Pincus, J.H., Alexander, C. and Freedman, M.H.: II. Allotypes and Idiotypes in Sera and Electrofocused Fractions. Eur. J. Immunol. In press.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Immunogenetics of Mouse Immunoglobulin

Previous Serial No.: Same

Principal Investigator: Rose Lieberman, M.A.

Other Investigators:

Project Subtitle I:	Dr. M. Potter, LCBGY, NCI W. Humphrey, Jr., LI/NIAID
Project Subtitle II:	Dr. M. Potter, LCBGY/NCI Dr. S. Rudikoff, LCBGY/NCI W. Humphrey, Jr. , LI/NIAID
Project Subtitle III:	
Project Subtitle IV:	Dr. M. Potter, LCBGY/NCI
Project Subtitle V:	Dr. M. Potter, LCBGY/NCI

Cooperating Units:
Dr. J. L. Claflin, Washington University, St. Louis, Mo.
Dr. J. M. Davie, Washington University, St. Louis, Mo.
Dr. M. J. Bosma, Inst. for Cancer Research, Fox Chase, Philadelphia, Pennsylvania
Dr. M. D. Scharff, Albert Einstein College of Medicine, Bronx, N.Y.

Man Years:

	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	1			1
Professional	6/12			6/12
Other	6/12			6/12

Project Subtitle I: Study of Immunoglobulin Determinants in Mice with Homologous Antisera to Determine Genetic Control of the Structure of Immunoglobulin Molecules

Project Description:

Objectives:

1. To prepare homologous anti-allotypic and idiotypic (myeloma-specific) antisera highly specific for determinants on various parts of the different chains of mouse immunoglobulin molecules.

2. To study genetic control of immunoglobulin determinants.

3. To use genetically controlled immunoglobulin markers in studies of antibody synthesis.

4. To determine if identical molecules, based on biologic activity and identification of Fab determinants, can be found in myeloma proteins from independently induced plasma cell tumors.

Methods Employed:

1. Preparation of immunoglobulin immunogens (Fc, Fab and light chains) from normal and plasma cell tumor bearing inbred mice.

2. Immunization of inbred strains of mice with specific normal or myeloma immunoglobulin molecules or fragments of immunoglobulin molecules (chains).

3. Identification of immunoglobulin determinants with homologous antisera using immunoelectrophoresis, Oudin and Ouchterlony gel diffusion methods, radioisotope tagging, precipitin inhibition method, hemagglutination inhibition techniques and immunoabsorbents.

4. Selective breeding of mice to determine linkage, allelism, independent assortment and crossover of genes. Production of coisogenic strains to propagate and isolate genetic markers in a known genetic environment. Chromosome mapping of genetic markers of immunoglobulin determinants with other genetic markers as coat color, etc., to facilitate easy identification of immunoglobulin genetic markers and also to determine the location on the chromosomes of genes controlling specific immunoglobulin determinants.

5. Development of techniques employing immunoglobulin determinants as genetic markers in problems of transplantation, histocompatibility, transfusion reactions, immunization and antibody synthesis.

Major Findings:

Heavy chain constant region (C_H) determinants have been identified on γ_1, γ_{2a}, γ_{2b} and γA myeloma proteins of BALB/c origin and a γA and γ_{2b} myeloma protein of C57BL origin (CB20). No determinants have been identified on γM or γG3 myeloma proteins. All the C_H antigenic determinants are controlled by closely linked genes. Twelve heavy chain antigenic groups have been identified; nine in inbred strains and three in wild mice.

In addition to C_H antigenic specificities, antigenic determinants present in other regions of the molecule, namely, the Fab or heavy chain variable (V_H) and possibly the light chain constant (C_L) regions have also been identified. Homologous antisera prepared with specific myeloma proteins

usually only identify the idiotypic determinants present on the Fab region of that myeloma protein used to prepare the antiserum. Occasionally cross reactions between idiotypes present on different myeloma proteins have been observed. This has been true of several different IgA myeloma proteins that bind phosphorylcholine. Other myeloma proteins that have the same or similar antibody activities are also being investigated for shared idiotypic specificities. Many myeloma proteins have been found to have anti-levan activity. Homologous antisera are presently being prepared to the idiotypic determinants of these proteins. In addition to myeloma proteins with known antibody activity (anti-phosphorylcholine) that share the same idiotype and appear to be structurally similar or identical, other myeloma proteins (HOPC1 and J5444) have been found that share cross reacting idiotypes that are not completely identical. Homologous antisera prepared to these specificities indicate that only one of two determinants present on the Fab of HOPC1 myeloma protein is shared by the S5444 myeloma proteins.

Significance to Biomedical Research and the Program of the Institute:

It is difficult to study the genetic control of the diversity of antibody using normal antibodies since these are mixtures of many types of molecules. Myeloma proteins are pure molecular forms of immunoglobulins and these can be obtained by experimentally inducing plasma cell tumors in some strains of genetically defined inbred mice. In addition, many of these proteins have been found to have antibody activity. Immunoglobulin determinants on both the common and variable regions of the heavy chains as well as conformational determinants involved in the antibody site provide tools to study the genetic control of the variability of antibodies. The finding of 5 identical myeloma proteins appears to indicate repetition of genetic mechanisms that control diversity of antibody production.

Project Subtitle II: Genetics of V-Regions

Project Description:

Objectives:

Identify and map as many genes controlling immunoglobulin variable regions (IgV genes) as possible in order to determine the frequency of recombination between various structural genes for antigen-binding sites of immunoglobulin and between these genes and genes controlling constant region structures.

Methods Employed:

Fab idiotype determinants present on myeloma proteins of BALB/c origin are serologically identified by homologous antisera. Some of the myeloma proteins have been determined to have antibody activity including a substantial number which bind phosphorylcholine. The idiotype found on

the Fab of a phosphorylcholine binding IgA myeloma protein, TEPC 15 (T15), was found to be present in normal serum of BALB/c. A survey of inbred, congenic and recombinant strains for the presence or absence of the T15 idiotype in their sera was done. In addition, normal sera of inbred strains were absorbed with phosphorylcholine conjugated to Sepharose beads to determine if molecules carrying the T15 idiotype could be removed.

Cross-reacting idiotypic determinants present on myeloma proteins whose antibody activity has not yet been characterized have also been serologically identified with homologous antiserum. A cross-reacting idiotypic specificity has been found on two $\gamma 2a$ myeloma proteins (HOPC 1 (H1) and S5444) which have $\lambda 1$ light chains. Antisera prepared in SJL/J mice separately to these two different BALB/c $\gamma 2a\lambda$ myeloma proteins identified allotypic specificities ($\gamma 2a$-Fc), idiotypic specificities (unique for each immunizing protein) and cross-reacting idiotypic specificities (found on both $\gamma 2a$ myeloma proteins).

The antisera made specific for the cross-reacting idiotype by absorption with a $\gamma 2a$, κ myeloma protein were then reacted with the non-immunizing cross-reacting $\gamma 2a\lambda$ myeloma protein coupled to sheep red blood cells (SRBC) in the hemagglutination inhibition system using normal BALB/c serum as the inhibitor.

Major Findings:

The T15 idiotype is absent in germ-free BALB/c but appears when the mice are conventionalized. A survey of normal sera of inbred strains for the T15 idiotype showed it to be present in BALB/c, 129, C57L, C58 and ST and absent or in low levels in CBA, C3H, C57BL/6, C57BL/ka, C57BL/10, SJL, B10.D2, DBA/2, RIII, A, AL, AKR, NAB and NH inbred strains of mice. The T15 idiotype is associated with some but not all strains carrying the IgC_H allotypes found in BALB/c. Linkage of genes controlling the T15 idiotype in normal serum to the IgC_H locus of BALB/c was demonstrated in F_2 progeny of a BALB/c and C57BL cross in Bailey's recombinant inbred strains (CxBD, CxBE, CxBH, CxBI, CxBJ, CxBK) and in CB20 congenic strains. Among these strains, only those possessing the IgC_H locus of BALB/c including the F_2 progeny consisting of BALB/c homocygotes and BALB/c/C57BL heterozygotes, and the CxBG and CxBJ recombinants showed the T15 idiotype.

The HI-S5444 idiotype has also been found in normal serum of some inbred strains while absent in others. The distribution pattern of the HI-S5444 idiotypic marker differs from that of T15. Normal BALB/c serum had an inhibitory titer equivalent to that of 38 to 75 µg/ml of HOPC1 or S5444. A survey for the HOPC1-S5444 idiotype in normal serum from other inbred and hybrid strains was made. Three phenotypes, high (log 2 mean titer 6-8), intermediate (log 2 mean titer 1-3) and low (log 2 mean titer < 1) were found. In the high phenotype were BALB/c and the Bailey RI strains CxBG and CxBJ; in the intermediate group were CBA, RF, DBA/1, I, A and AKR, and in the low group were BRSUNT, C57BL/10, C57BL/6, LP, RIII, NZB, CE, BAB14, CB20, CxBD, CxBE, CxBH, CxBI and CxBK. The cross-reacting HOPC1 - S5444 idiotype was associated with some but not all the strains in three IgC_H (allotype) groups (a^1, a^3 and a^4) indicating an unusual strain distribution of this phenotype.

The presence of the high HOPC1-S5444 idiotype in the Bailey CxBG and CxBJ strains, which are of a^1 allotype and the low level in the congenic CB20 and BAB14, which are of a^2 allotype and in all of the Bailey RI strains of a^2 allotype strongly suggests linkage of the genes controlling the HOPC1-S5444 idiotype to the IgC_H gene complex. Accordingly, the sera of (BALB/c X C57BL/6)F_1 x BALB/c progeny were examined for the presence of HOPC1-S5444 idiotype. 39 homozygous a^2a^2 (allotype of C57BL/6) lacked the idiotype while 39 a^1a^2 progeny (heterozygous for BALB/c and C57BL/6 allotype) had intermediate levels of idiotype. This result further supports the linkage of the genes controlling the HOPC1-S5444 idiotype and the IgC_H allotypic specificities, although it suggests incomplete penetrance of the trait in heterozygotes.

Anti-HOPC1-S5444 idiotype antisera were partially inhibited by other λ-type myeloma proteins including a $\mu\ \lambda_1$ (MOPC 104E), an $\alpha\lambda_1$ (J558) and a λ_1 Bence-Jones protein (RPC-20) but not by an $\alpha\lambda_2$ (MOPC 315). Further investigation of the λ-related specificity is underway.

Significance to Biomedical Research and the Program of the Institute:

The identification of genes controlling immunoglobulin variable region determinants permits studies of mechanisms of antibody diversity and formation of antibody molecules.

Project Subtitle III: Clonal Nature of the Immune Response to Phosphoryl Choline: Idiotypic Specificity and Binding Characteristics of Anti-Phosphorylcholine Antibodies

Project Description:

Objectives:

Idiotypic specificity and binding characteristics of normal anti-phosphorylcholine (PC) antibodies was studied at the level of the antibody-producing cell (PFC) and compared to those exhibited by four different PC-binding myeloma proteins, HOPC8, McPC603, MOPC 167 and MOPC511 each of which possesses a distinct idiotype which do not specifically cross-react with one another and also have different binding affinities for PC glyceryl phosphorylcholine (GPC) and choline (C).

Methods Employed:

BALB/c and A/J mice were immunized with 10^8 D. pneumoniae organisms (R36) and characteristics of the PC specific binding cell(ABC), and plaque-forming cell (PFC) responses were assessed by inhibition of binding and lysis by PC and its analogies by a antiserum specific for idiotypes of 4 different PC-binding BALB/c myeloma proteins (H8, M603, M511 and M167).

Major Findings:

The anti-PC antibody was solely of the IgM class. BALB/c and A/J but not CBA showed a restriction in the affinity heterogeneity of the antibody they produced. BALB/c and A anti-PC antibody bound H8 similarly but only BALB/c carried the T15 idiotype present on T15 myeloma protein. These results are in accordance with those previously shown that PC receptors on cells show the same class specificity and idiotypes as the secreted anti-PC antibody. The present data suggest a direct link between secreted anti-PC antibody and PC receptors on antigen binding cells.

Significance to Biomedical Research and the Program of the Institute:

These experiments provide a model system through which the relationship of receptors on precursors of antibody secreting cells and of the secreted products of their descendents can be clearly analyzed.

Project Subtitle IV: Identification of Genetic Markers in BALB/c Myeloma Cell Lines Treated with Mitogenic Agents

Project Description:

Objectives:

Separate cell lines of MPC11, a γ_2b producing myeloma line, were treated with various mitogens and were then examined for the idiotypic and allotypic markers on the immunoglobulin they produced. Some cell lines exhibited a switch in the class of immunoglobulin being produced from γ_2b to γ_2a. Our goal was to correlate structural and serologic information in the study of idiotypic and allotypic markers expressed by mitogen-treated cell lines of MPC11 myeloma tumor.

Methods Employed:

Homologous antisera was prepared to the MPC11 protein obtained from ascitie fluid of tumor bearing mice. Antisera were made specific to either the MPC11 idiotype or to the $H^{9,11,22}$ allotypic determinants. These antisera were employed to examine the secreted immunoglobulins from various MPC11 mitogenic cell lines. In addition, other homologous antisera that were used to determine allotypic determinants of the γ_2a class.

Major Findings:

Some cell lines treated with mitogens no longer produced detectable amounts of allotypic determinants; in others production to both allotypic and idiotypic determinants appeared to cease. One of the cell lines that appeared to switch from γ_2b to γ_2a class carried the allotypic $G^{1,6,7,8}$ determinants of the γ_2a class while still retaining the original MPC11 wild type idiotype. In addition, some cell lines in which the COOH region

of their Ig was lost appeared to lose the $H^{9,11,22}$ allotypic determinants. Sequence studies are presently underway to correlate serologic results with structural findings.

Significance to Biomedical Research and the Program of the Institute:

These studies may give us some insight into the ontogeny of immunoglobulin structure and genetic control.

Project Subtitle V: Role of Regulatory Genes in the Expression of V_H and C_H Immunoglobulin Phenotypes

Project Description:

Objectives:

1. To relate the generation of antibody diversity to the inheritance of an altered library of Ig genes.

2. To test the hypothesis that differentiation of stem cells into Ig-bearing precursor cells is under the control of thymus-dependent regulator genes.

3. To determine the kind of variant Ig molecules that may derive from monoclonal proliferation of Ig-producing cells.

Methods Employed:

Mice congenic to BALB/c except for the immunoglobulin genes of the C57BL allotype were used as the biologic material for these studies. A different number of backcrosses to BALB/c preceded the derivation of such congenic strains (CB-17, CB-23 and CB-26) before they were made homozygous for the C57BL allotype. As a results of genetic recombination, we would expect the C57BL chromosomes to acquire many BALB/c genes including BALB/c Ig genes. Thus the hypotheses we tested was: 1) will continued backcrosses result in diminished C57BL Ig genes on the C57BL chromosomes at the expense of an increasing number of nontranscribed BALB/c recombinant genes. 2) Is the lack of expression of the BALB/c gene due to the absence of specific regulator genes that may be under thymus control? 3) Does the breeding of congenic CB strains select against the acquisition of BALB/c regulator genes?

We will attempt to show such gene differences by testing the immunocompetence of CB mice to different antigenic determinants as a function of age. We will also substitute for putative immunologic deficiencies by reconstituting thymectomized x-irradiated CB mice with thymocytes of congenic strain CAL 20 which expresses only the AL allotype.

Major Findings:

In certain instances Ig immunoglobulin allotypic determinants of the BALB/c appeared in the serum of CB-17 mice which are homozygous for the C57BL/Ka allotype. The appearance of his hidden allotype was usually transient and only associated with the γ_2a class.

Significance to Biomedical Research and the Program of the Institute:

These studies lead to a further resolution of Ig structural genes and factors controlling their expression.

Publications:

Lieberman, R., Potter, M., Mushinski, E.B., Humphrey, W., Jr. and Rudikoff, S.: Genetics of a new Ig V_H (T15-idiotype) marker in the mouse regulating natural antibody to phosphorylcholine. J. Exp. Med. 139: 983-1004, 1974.

Claflin, J.L., Lieberman, R. and Davie, J.M.: Clonal nature of the immune response to phosphorylcholine. I. Specificity, class and idiotype of phosphorylcholine binding receptors on lymphoid cells. J. Exp. Med. 139: 59, 1974.

Claflin, J.L., Lieberman, R. and Davie, J.M.: Clonal nature of the immune response to phosphorylcholine. II. Idiotypic specificity and binding characteristics of anti-phosphorylcholine antibodies. J. Immunol. 112: 1747-1756, 1974.

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Levy, N.L., Snyderman, R., Ladda, R.L. and Lieberman, R.: Cytogenetic engineering in vivo--restoration of biologic complement activity to C5--deficient mice by the intravenous inoculation of hybrid cells. Proc. Nat. Acad. Sci. 70: 3125, 1973.

Serial No. NIAID - 148
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Genetic Control of Antibody Responses

Previous Serial No.: 148

Principal Investigators: Miss Rose Lieberman, M.A. and Mr. William Humphrey, Jr.

Other Investigators: Project Subtitle I: Dr. William E. Paul, LI/NIAID
Project Subtitle II: Dr. G. Biozzi, Institut d' Immuno-Biologie, Paris, France
Dr. C. Stiffel, Institut d' Immuno-Biologie, Paris, France

Cooperating Units: Drs. G. Biozzi and C. Stiffel, Institut d' Immuno-Biologie, Paris, France; Drs. M. Sela and E. Mozes, Weizmann Institut Science, Rehovath, Israel

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	1			1
Professional	6/12			6/12
Other	6/12			6/12

Project Subtitle I: Genetic Control of Antibody Responses to Myeloma Proteins of Mice

Project Description:

Objectives:

It has been shown in the mouse that the immune response to a BALB/c $\gamma 2a^{K}$ type myeloma protein (MOPC173) is controlled by the immune response gene Ir-IgG. In mice with IgC_H allotypes different from BALB/c, the Ir-IgG gene was found to be linked to H-2 types b, bc, p, r, s and v not to a, d, k and q. The response of mice of the $H\text{-}2^{b}$ type and lack of response of $H\text{-}2^{a}$ mice permitted us the opportunity to map the Ir-IgG gene in the H-2 region by investigating the immune response of the $H\text{-}2^{a}/H\text{-}2^{b}$ recombinants that were available. These recombinants included B10.A (1R), B10.A (2R), B10.A (3R), B10.A (4R), and B10.A(5R). It was found that 3R, 5R and 4R gave a high response while 1R and 2R did not. On the basis of prior mapping of other

Ir genes, e.g., Ir-I and Ir-IgA, it was shown that Ir-IgG was located between the Ir-IgA, Ir-I complex and Ss-Slp. In that study we postulated that the allotype present on the C_H (Fc) region was recognized by specific thymus-derived (T) lymphocytes and that an immune response to the idiotype present on the Fab could only be elicited when the allotypes of the immunized mouse differed from BALB/c. On these premises, irrespective of the differences in the V_H regions (idiotypes), the same H-2 linked Ir gene should be involved in responses to all myeloma proteins of the same class. To test this view, the immune response to six different BALB/c γ2a myeloma proteins was investigated in inbred, H-2 congenic and recombinant strains.

Methods Employed:

The immune response to six different myeloma proteins of BALB/c origin was determined. Four of the γ2a myeloma proteins were of the kappa type (MOPC173, LPCI, UPC10 and RPC5); two were of the lambda type (HOPC1 and S5444). All the six myeloma proteins have the same C_H region allotype determinants ($G^{1,6,7,8}$). Each of the four kappa type proteins has its own unique idiotypic specificity which does not cross-react with that of any of the others. The two lambda types in addition to possessing unique idiotypic specificities, also have cross-reacting idiotypic determinants.

Major Findings:

Among the inbred strain of various H-2 types and Ig allotypes the immune response for the six γ2a myeloma proteins was remarkably consistent. No antibody response was observed in mice with the same allotype as BALB/c, including one strain of the H-2^b type. In strains of mice with allotypes different from BALB/c, a high immune response (mean log 2 HI titer 8 - > 12) was found in H-2 types b, r, s and v for 2 or 3 of the k types and for 1 or both of the lambda types. A low or absent immune response was found in H-2 types a, d, k and q for at least 2 of the 4 kappa types and for one of the lambda types. One notable exception was the high response of RF (H-2^k) to UPC10, a kappa type γ2a protein. Among the kappa types, a high response was found in H-2^b (B10) and a low response in H-2^a (B10.A). Among the H-2^a/H-2^b recombinants, 1R and 2R gave a low response while 4R and 5R gave a high response. The response of 4R while present, was always less than that of 5R or B10. The data indicate that regardless of the V_H region of the myeloma protein, the same Ir gene (Ir-IgG) is employed in the immune response to κ type γ2a BALB/c myeloma proteins.

The findings with the λ type γ2a proteins were quite different. Firstly, the results on repeated experiments revealed a marked variability among individuals within a strain, a finding not observed with the κ type proteins. For S5444 all the B10 mice gave a high response whereas for HOPC1 only 3/22 gave a high response in three separate experiments using different preparations of HOPC1. Both the S5444 and HOPC1 gave low responses in the recombinants 2R, 4R and 5R. However, there were some inconsistencies in these latter groups. Among the 4R, 2/18 gave a good response and among the 5R, 2/23 gave a good response to HOPC1. Despite this

variability, it was still clear that the immune response to HOPC1 was linked to the H-2 complex. Thus, all individuals of the B10.M strain gave a very high immune response (log 2 H-1 mean titer > 12).

The data support the concept that the genetic control of responsiveness to γG (γ2a) κ myeloma proteins involves recognition of constant region allotype associated determinants. This most likely involves antigen recognition by T lymphocytes, but out data do not establish such a role for T lymphocytes. However, one strain (RF) responded to UPC10, but not MOPC173 or LPC1, suggesting in this case a recognition limited to a unique antigen of UPC10.

The situation of the lambda γ2a proteins is much less clear, although it seems possible that an Ir gene distinct from, although linked to, that controlling responsiveness to κ γ2a proteins may be involved.

Significance to Biomedical Research and the Program of the Institute:

Histocompatibility-linked Ir genes play an important role in antigen-recognition by T lymphocytes and in the control of susceptibility and resistance to a variety of diseases. The mechanisms by which these critical functions are mediated is still unknown; their delineation should be important in specific manipulation of the immune response. In addition, Ir genes also serve as genetic markers to study gene products at the cellular level.

Project Subtitle II: Allotype Linked Ir Genes

Project Description:

Objectives:

Two lines of mice originally chosen on the basis of responsiveness to sheep erythrocytes have been bred for 30 generations. One has a low response to sheep erythrocytes (SRBC) and possess immunoglobulin of the $IgG^{3,5}$ allotype; the other has a higher response to SRBC and possess immunoglobulin of the $^{2}G^{1}$ or $G^{1\ of\ 2}$ allotype. F_1 progeny from high and low matings were backcrossed to either the low or high responders to analyze the control of immune response to SRBC. It was established that genes controlling immunoglobulin allotype (or genes closely linked to allotype genes) were of importance in the responsiveness of these mice. A further analysis of genetic linkage in these mice may allow delineations of the types of genes concerned with the control of immunoglobulin synthesis.

Methods Employed:

F_1 progeny of a cross of strains of mice selected for low or high responses to SRBC for more than 30 generations were backcrossed to either low or high responders and then progeny were tested for Ig allotypes, H-2 types, immune response to SRBC, SIII and other antigens and for immunoglobulin levels.

F_2 progeny of a high and low responders were similarly tested.

Major Findings:

There appears to be a disappearance or a low level of the allotype marker G^5 that was formerly found linked to the G^3 in the "Low" responder strains. These mice have now been selectively bred for low and high response to SRBC from over 26 generations and the loss of the G^5 allotype suggests the possibility of some regulatory mechanism linked to allotype expression.

Significance to Biomedical Research and the Program of the Institute:

The genetic control of the immune response and antibody synthesis is concerned in the understanding of resistance and suceptibility to disease. This system may provide a model for analysis of some form of hypogammaglobulinemia and hyporesponsiveness.

Serial No. NIAID - 149
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Structural and Activity Studies on Immunologically Important Cells and Proteins

Previous Serial No:

Principal Investigator: Dr. Myron J. Waxdal

Other Investigators: Project Subtitle II: Dr. Rose Mage, LI/NIAID
Dr. Salvatore Carta, LI/NIAID
Project Subtitle IV: Mrs. V. Vinton, DCRT
Mr. LeRoy Brown, DCRT
Mr. Bob Martino, DCRT
Mr. Dave Songco, DCRT
Project Subtitle III: Dr. Joerg G. Nagel,

Cooperating Units: George Washington University Medical Division

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to	Guest Workers	Total
Total	2 9/12	2 3/12		5
Professional	1 3/12	2 3/12		3 6/12
Other	1 6/12	0		1 6/12

Project Subtitle I: Lectins and their interaction with cells

Project Description:

Objectives:

The initation of the immune response is triggered by the specific interaction of antigens with cell surface receptors on a small percentage of immunocompetent cells. Larger percentages of these cells are stimulated by certain mitogens. In this project the ability of the 5 pokeweed mitogens (Pa-1 to Pa-5) to stimulate mitosis and immunoglobulin production in different populations of murine lymphoid cells is investigated to increase our knowledge of the early events in cell differentiation and mitosis.

Methods Employed:

Pa-1 through Pa-5 were prepared from pokeweed roots by salt fractionation and column chromatography. The ability of murine lymphocytes to respond, *in vitro*, to these mitogens was determined by assaying the incorporation of radioactive precursors into DNA, protein and specifically isolated immunoglobulin. The lymphocyte populations employed were spleen cells and thymocytes from normal BALB/c mice, spleen cells from adult thymectomized, lethally irradiated BALB/c mice reconstituted with anti-θ treated bone marrow cells (TXBM-mice) and spleen cells from athymic nude mice and their heterozygotic littermates.

Major Findings:

All five of the pokeweed mitogens stimulate mitosis in normal spleen cells and thymocytes. The responses of the thymocytes indicated that all of the pokeweed mitogens can stimulate mitosis in T cells. Pa-2 is the most active of these mitogens, stimulating spleen cells to the same extent as the T cell mitogen concanavalin A and stimulating thymocytes to a level 3-fold that on concanavalin A. Pa-1 was the only mitogen to stimulate TXBM spleen cells to the levels found with cells from normal spleens. The other pokeweed mitogens and concanavalin A induced weaker responses. While these results suggest that Pa-1 may be a B-cell mitgen as well as a T cell mitogen, the response to concanavalin A indicates the presence of residual T cells and makes it impossible to conclude whether the response to Pa-1 and the lower response of the other pokeweed mitogens are due to their possible B cell activities or to the presence of contaminating T cells. The data obtained using spleen cells from athymic nude mice resolved this ambiguity. These cells responded only to Pa-1; the other pokeweed mitogens and concanavalin A either showed no stimulatory activity or killed the cells. These data indicate that in the murine system Pa-1 is both a T cell and a B cell mitogen and that the other pokeweed mitogens are T cell mitogens. It was also determined that Pa-1 was the only pokeweed mitogen to stimulate protein synthesis and immunoglobulin production in the B spleen cells from athymic nude mice.

The difference between the mitotic and immunologic stimulating properties of Pa-1 and the other 4 pokeweed mitogens is reflected in the difference in physico-chemical properties of Pa-1 and the other mitogens. Pa-1 differs markedly in these properties; it has a subunit molecular weight of 22,000 Daltons, but apparently exists as an aggregate or polymer in aqueous solutions. The 4 other mitogens all appear to be monomers. Pa-1 also differs noticeably from the other proteins in its amino acid composition, and has few disulfide bonds. In contrast, Pa-2, Pa-3, Pa-4 and Pa-5 are similar to one another in composition and have many disulfide bonds.

Significance to Biomedical Research and the Program of the Institute:

The dual activity of Pa-1 is unique; there is no other definitive case in which a mitogenic protein has activities directed towards both the B and the T classes of lymphoid cells. The polymeric state of Pa-1 may be the key to its ability to stimulate B cells; the requirement of multiple interactions between the B cell immunoglobulin receptors and specific antigens for stimulation is well known. The multimeric state of Pa-1 may fulfill the same requirements, but stimulate a much higher percentage of cells than possible with antigens requiring specific immunoglobulin receptors. Studies with this model system will further our understanding of the initiation of the immune response.

Proposed Course of Project:

Further detailed studies on the interaction of Pa-1 and other mitogens with cell surfaces will be undertaken. These studies will provide information about the type and distribution of the cellular receptors as well as the interaction between these receptors and the lectins. A knowledge of these features is necessary for our further understanding of cell stimulation.

Project Subtitle II: Structural Studies of Immunoglobulins

Project Description:

Objectives:

To study the salient features of the antibody combining site and the structures of different antibodies which recognize the same antigen. In particular, to compare the covalent structures of the heavy chain variable regions of two antibodies, produced in the same rabbit, against the same carbohydrate antigen.

Methods Employed:

The antibodies were isolated by ion-exchange chromatography and their heavy and light chains separated by gel filtration. The heavy chains were cleaved at methionyl residues with cyanogen bromide and the resulting fragments separated by gel filtration in strongly dissociating solvents. Certain of their fragments have been subjected to sequence analysis using the Beckman Model 890C segneator.

Major Findings:

The cyanogen bromide fragments of these two antibodies have been isolated and characterized by molecular weight, NH_2- and COOH-terminal residues and amino acid analyses. Incomplete cleavage, or possible microheterogeneity at certain methionyl positions, and strong aggregation by some of these fragments, has been particularly troublesome. The

staphylococcus sp. Antibodies of higher activity were obtained from the immunized rabbits as compared to the infected patients, and form precipitates with both the organisms and very small concentrations (∿50 ng) of the extracted β-techoic acid.

Significance to Biomedical Research and the Program of the Institute:

The completion of this project provides the clinician with a simple, quick and accurate tool for detecting $^{\alpha}$ and β forms of techoic acid and may provide a test useful in diagnosis of the early stages of staphylococcal endocarditis and other staphylococcal infections.

Proposed Course of Project:

The isolated antibodies will be adsorbed to tanned sheep erythrocytes and covalently bonded to suitable beads. These materials will be used to develop a method of detecting small amounts of the free antigen in the sera and urine of infected patients.

Project Subtitle IV: Laboratory Automation and Data Processing

Project Description:

Objectives:

To achieve maximal utilization of several mechanized analytical instruments used in pursuing the objectives of LI/NIAID by automatic data acquisition, data reduction and calculation, data display and instrument monitoring. To develop new automated procedures for structural studies on cells and proteins, which will reduce the time, effort, and mistakes inherent in coventional procedures.

Methods Employed:

A PDP-11 mini-computer is used to acquire analogue and digital data from the laboratory's instruments through NIH fabricated interfaces. Data from scintillation counters is transmitted to the computer by punched paper tape. The raw data is reduced and stored in the PDP-11's disc storage, or transferred by data phones to DCRT's time shared PDP-10. This data is held for further processing under the operator's control or will be used by the computer for online decisions in the control of the atuomated instruments. Operator interaction is by keyboard and cathode ray tube display of the processed data.

Major Findings:

The computer has been programmed to accept and process data from 4 channels of a multiplexed spectrophotometer. This has included the writing of an extensive real time data acquisition and processing executive program

fragments have been ordered and mapped onto the heavy chains and were found to account for the entire chains.

Two of the fragments from the major antibody, arising from the variable region, were subjected to sequence analysis. Our tentative sequences in these regions are in basic agreement with partial sequences of other rabbit immunoglobulins of the same allotype determined by others, but differences occur at certain positions, as expected in these variable region.

Significance to Biomedical Research and the Program of the Institute:

These structural studies help define the chemical basis of the specificity of antibodies and provide information on the nature of the genes and genetic mechamisms which control antibody structure and the immune response. In a broader sense, the elucidation of the chemical differences in antibodies which recognize the same antigen will shed light on redundency and compensation in protein structures and the influence of primary structure on tertiary structure and activity.

Proposed Course of Project:

The sequence analyses of these two antibodies will be continued to define the pertinent chemical differences in their structures.

Project Subtitle III:

Objectives:

To investigate the structure and activity of proteins possessing a special property to make them of current interest. To isolate pure antibodies to a component of the cell wall Staphylococcus sp. and to use these antibodies in a clinical diagnostic test to detect staphylococcal endocarditis in its early stages.

Methods Employed:

The A and B forms of techoic acid were isolated from Staphylococcus sp. and purified. These antigens were coupled to various bead supports by the usual procedures. Human antibodies to these antigens were isolated from the sera of patients with staphylococcal endocarditis by affinity chromatography. Antibodies were also isolated in the same manner from the sera of rabbits which had been immunized with the killed organisms.

Major Findings:

Antibodies with specificities for the α and β forms of techoic acid have been isolated from human and rabbit sera. These antibodies appear to be of high purity and specific for one form of the antigen from

(RTDAPE). RTDAPE has been structured to function as an executive, running data acquisitions sub-routines and data reduction sub-routines on a time dependent basis from a task table. RTDAPE also may call processing programs as directed by the task table or by the operator. The data acquisition priority programs are continually executed even though other other programs may be running. Fortran programs for the acquisition of two channels of data from an amino acid analyzer and for the operator directed processing of this data via the CRT have been written and are being used. A Fortran program for the processing of data from the scintillation counter on paper tape has also been written and is being used by several members of LI. The necessary programs for communications between the PDP-11 and the time shared PDP-10 have also been written and may be used to transfer data between the minicomputer (PDP-11) and the larger computer in DCRT.

The necessary hardware interfaces between the computer and the spectrophotometer, fraction collectors, and amino acid analyzer have been designed, constructed and completed.

Significance to Biomedical Research and to the Program of the Institute:

The writing of the RTDAPE program was a slow but necessary step in the creation of the laboratory computer system. The acquisition and reduction modules for the various instruments now can be written and linked into this executive. RTDAPE will also call and monitor data processing programs and their interactions with the operator, such that the higher priority programs will continually function.

Now the automation and control of laboratory instrumentation can proceed and will increase the productivity of the instruments and the investigators using them. The alleviation of much of the drudgery of manual analytical procedures will allow the highly trained laboratory personnel to concentrate on higher duties and to attack more difficult problems.

Proposed Course of Project:

Hardware and software development and testing will continue. The Fortran acquisition program for the amino acid analyzer will be rewritten in assembly language to run under RTDAPE. The output from a gas liquid chromatograph will be interfaced to the computer for the identification and quantitation of PTH-amino acids obtained from sequence studies. Digital output from the computer will be utilized to control certain of the laboratories analytical instruments. The processing of data from the scintillation counters will be improved and modified to provide this service to all members of LI.

Publications:

Waxdal, M.J.: Isolation, characterization and biological activities of 5 mitogens from pokeweed. Submitted to Biochemistry.

Waxdal, M.J. and Basham, T.Y.: The B and T cell stimulatory activities of multiple mitogens from pokeweed. Submitted to Nature.

ROCKY MOUNTAIN LABORATORY

HAMILTON, MONTANA

July 1, 1973 through June 30, 1974

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PHS-NIH

Summary Statement

Rocky Mountain Laboratory

Hamilton, Montana

July 1, 1973 through June 30, 1974

Currently, our research program is directed primarily toward problems in 5 major areas—slow viral diseases, immunology, rickettsiology, medical entomology and acarology, and cancer immunotherapy. Only minor changes were made in the direction of programs following an overall review by the Board of Scientific Counselors in August 1973. Only a few staff changes were made during the year. Dr. Thomas Meyer and Dr. Francis Abinanti, who were both engaged in research on cancer immunotherapy, left the laboratory, and Dr. Donald Granger joined the staff as a Research Associate. Drs. Robert Philip and Robert Gerloff reoriented much of their programs from research on chlamydiae to rickettsial diseases.

Progress on major projects is presented in the text of the report. Many of the major findings were published in 71 publications, of which 23 are still in press.

The following highlights of research findings deserve special mention:

Rickettsioses

During collaborative studies of spotted fever in the Southeast, one investigator submitted acute and convalescent phase serums from 46 of 58 patients who had tick-associated illnesses typical of spotted fever. However, only 37% of these patients had diagnostic 4-fold increases or significant antibody titer during convalescence. These findings strongly suggest a rickettsial agent other than Rickettsia rickettsii. During these same investigations, 2 rickettsia-like organisms were recovered from ticks taken from dogs and people. One found in both Dermacentor variabilis and Amblyomma americanum was related to R. rickettsii and was nonpathogenic for laboratory animals but grew in tissue cultures. The other, recovered from Rhipicephalus sanguineus and also related to R. rickettsii, was infectious for meadow voles, cross reacted with FITC-labelled anti-R. rickettsii conjugates, and induced strong protection in guinea pigs against challenge with virulent R. rickettsii. Serums of cases of spotted fever in the south will be tested serologically against these agents to determine whether they could be the cause of this human disease.

Additional attempts to cause a shift of cloned phase I Coxiella burneti to phase II in fertile hens' eggs failed. Similarly, it was impossible to cause a shift of phase II to phase I by guinea pig passage of cloned phase II organisms, which could not be passed in guinea pigs more than 2 times.

Apparently, C. burneti exists in nature as a mixture of phase I and phase II, with the former predominant in animal tissues.

Major methodological advances were made in the mass cultivation of R. rickettsii in mouse cell spinner cultures, in which the yield per gm of tissue exceeded that in fertile hens' eggs by at least 70-fold. Rickettsiae purified from tissue cultures or fertile hens' eggs by zonal centrifugation were shown to be highly immunogenic in guinea pigs and serologically reactive in various tests.

Rabies

In further studies on the effects of hyperthermia on the progression of rabies infection in mice, we found that high ambient temperature raised the temperatures of infected mice, but not those of normal mice. Reduction in mortality and increase in abortive infection were proportional to the total time infected mice were exposed to increased ambient temperature. Exposure during the incubation period delayed onset of illness.

Chronic progressive rabies has been demonstrated in a cat that survived infection 3 years ago. During repeated monitoring of the antibody content in its serum and cerebrospinal fluid, we observed that titers increased when the condition of the cat progressively deteriorated. Because of hind-limb paralysis, fasciculation of thigh muscles, and general deterioration, the cat was killed; histologic changes characteristic of progressive disease were seen in its brain.

Arthropod-borne Viruses

Results of ecologic studies of Tuleniy virus at Yaquina Head, Oregon, indicated that murres are commonly infected—19 of 24 were seropositive. However, adult and baby murres and seagull chicks experimentally inoculated with Tuleniy virus developed only low level viremias of brief duration. Likewise, fresh chicks and domestic ducks were shown to be poor donors of circulating Tuleniy virus. Considering the low levels of viremia in these birds and the prevalence of antibodies among murres, one wonders how the virus is circulated between ticks and birds during their brief 3-month nesting period on rocky islands of the West Coast.

Some investigators contend that Tuleniy virus is the progenitor of Japanese B encephalitis (JBE) virus—essentially a strain of JBE isolated from ticks. Both Hsu's and Schneider's lines of Culex tritaenorhynchus cells supported rapid increases of 2 strains of JBE virus, but not that of Tuleniy virus. Furthermore, in 4 experimental attempts to infect this species of mosquito, a principal natural vector of JBE, it was shown to be refractory to Tuleniy virus. After these mosquitoes were inoculated intrathoracically, their virus content declined rapidly and virus could not be detected after day 7 postinoculation.

The application of the scanning electron microscope (SEM) to taxonomic studies on ticks has revealed specific characters undetectable by light and phase microscopy. A species of a remarkable new genus (possibly family) of Argasidae is presently being described with the aid of SEM. This tick was collected from a bat cave in Campeche, Mexico. About 40 African species of the genus Rhipicephalus are now being studied by SEM. The sanguineus group of this complex has been particularly difficult to characterize.

Pathogenesis and Immunology of Slow Viral Diseases

Several observations on slow viral diseases are noteworthy. Additional data support earlier findings that lambs likely acquire scrapie virus via the oral route early in postnatal life. On the basis of studies on experimentally infected goats, it was believed that the goat was an aberrant host from which virus was never released. However, in 2 naturally infected goats, the distribution of virus in non-neural tissues, especially gut, was similar to that seen in Suffolk sheep. The recent development of encephalopathy in one of 2 mink inoculated with brain of Suffolk sheep naturally affected with scrapie suggests that this disease in ranch mink is indeed scrapie. The production of scrapie in 10 of 19 goats inoculated with mink encephalopathy virus provides additional support to this thesis.

The antibody response of mink to the Porter strain of Aleutian disease (AD) virus has been measured accurately by counter immunoelectrophoresis. The precipitating antigen has been found to be associated with the intact virion in precipitin lines examined by electron microscopy. In sapphire mink inoculated with the Pullman strain, antibody response seems tantamount to subsequent disease, but in pastel mink antibody is produced without overt disease or measurable changes in serum gamma globulin. The antibody response to parallel inocula was higher and persisted longer in sapphire than in pastel mink. In associated studies on changes in Ig content during the course of AD, we found that the γM remained unchanged, γA increased tremendously in pastels and that the γG increased comparably in both color phases. The B_1C content declined in sapphire but increased 203% in pastels. About 50% to 60% of the Ig-positive B cells of mink were found to have a receptor for C_3. In a study of the distribution of these B lymphocytes in lymphoid organs and cellular infiltrates in AD-affected mink, we demonstrated that infiltrates in the kidneys of affected mink, as well as diseased glomeruli, bound complement.

Basic Immunology

In continued studies on the role of cell-mediated immunity in herpes simplex virus (HSV) infections it was determined that "activation" of leukocytes sensitized to a nonrelated antigen had a profound effect on their ability to inhibit HSV replication in cell cultures. This reaction was shown to be dependent on the degree of sensitization, concentration of activating antigen, and the number of leukocytes per target cell.

Histamine sensitization of mice induced by Bordetella pertussis extracts is associated with increased γE-globulin production. Because Trichinella spiralis infections in mice are known to cause increased γE-globulin production, the possibility that infection with this nematode may likewise induce histamine sensitivity in mice was explored. Oral infection of mice with 50 to 100 larvae induced a prolonged sensitization, beginning about the 14th day after exposure. The LD_{50} of histamine in infected mice was 0.18 mg whereas that in normal mice was 15 mg. The γE-globulin induced by T. spiralis blocked the PCA activity of γE having specificity to a heterologous antigen.

Role of Mycobacterial Constituents in Cancer Immunotherapy

Further progress has been made in clarifying questions about the role of various constituents of mycobacterial cell walls in cancer immunotherapy. The free lipids extracted with organic solvents from cell walls yielded 5 fractions when subjected to microparticulate gel chromatography—1) mono- or oligomeric peptidoglycolipid (MA-AG-MA), 2) P1, 3) P2, 4) P3, and 5) P4. The first is similar to cell wall skeleton, except that its peptidoglycolipid is polymeric. MA-AC-MP and cell wall skeleton will suppress tumors, but will not cause regression unless P3 is added. However, neither are effective unless they contain at least 1% tuberculoprotein, which represents the specific antigen to which delayed hypersensitivity or cell-mediated immunity is directed. Indeed, the efficacy of cell wall skeletons containing less than 1% protein could be enhanced by the addition of PPD.

The lipid-free (no mycolic acid), water-soluble mycobacterial adjuvant and antitumor factor (MAAF) described by Hiu regressed tumors but only when mixed with P3. MAAF alone could enhance tumor growth. This preparation and acetylated cell walls are effective adjuvants for humoral antibody production against unrelated antigens, but by themselves, neither will elicit a Koch reaction in the skin of guinea pigs. Conceivably, their enhancement of tumor growth is due to the humoral response to specific transplantation antigens, wherein antibodies interfere with the action of cell-mediated immunity.

Immunoprophylaxis against Tuberculosis

The protein requirement for efficacy of cell wall (CW) skeleton in regressing tumors prompted further study of the role of tuberculoprotein in immunoprophylaxis against tuberculosis. Surprisingly, a combination of P3 and PPD (75 μg each) produced a vigorous granulomatous inflammation in the lungs of mice and protected them against tuberculosis. Neither PPD nor P3 alone were active. So far, the most potent P3-protein combination was one made with protein extracted from BCG CW with aqueous butanol. Granuloma formation and protection were obtained even when P3 was combined with the dialyzable portion of peptides (M.W. <10,000) split from tuberculoprotein by acid hydrolysis.

In collaboration with Drs. Salvin and Youngner, we made further investigations on the role of mycobacterial fractions in the *in vivo* production of migration inhibition factor (MIF) and interferon. A combination of P3 and PPD was found as effective as cell walls for sensitizing mice for the production of MIF. The addition of CW skeleton did not augment the response and neither P3 nor PPD alone was effective. The role of P3 in inducing cell-mediated immunity is further illustrated in this test system by the inactivity of *Mycobacterium smegmatis* CW, which are free of P3, and the activity of *M. kansasii* CW, which contain P3.

Microbial Endotoxins

The specificity of the limulus test for endotoxin was evaluated more critically in assays of typical bacterial endotoxins, extracts of polysaccharide-deficient mutants, anomalous lipopolysaccharides (LPS) from Brucella and Thiobacillus, synthetic polynucleotides, and extracts of *Candida albicans*. Among anomalous LPS, there was little correlation between the limulus test and other standard tests. One LPS from *Thiobacillus ferrooxidans*, which was weakly pyrogenic, was as potent in the limulus test as LPS from *Salmonella enteritidis*. Also, extracts of Candida devoid of pyrogenicity for rabbits clotted limulus lysate. Its unusual sensitivity for substances other than typical bacterial endotoxin will limit its application for testing pharmaceuticals for contaminating endotoxins.

Serial No. NIAID-170
1. Rocky Mountain Laboratory
2. Office of the Director
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Office of the Director

Previous Serial Number: Same

Principal Investigator: H. G. Stoenner, Director

Other Investigator: R. N. Philip, Assistant Director

Cooperating Units: None

Man Years:	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	42			42
Professional:	1 2/12			1 2/12
Other:	40 10/12			40 10/12

Project Description:

The general administration of the laboratory, supervision of research programs, personnel management, budget and fiscal operations, and research support services are combined in this project.

Honors and Awards:

Dr. H. G. Stoenner

Continued as a Reviewer for American Journal of Veterinary Research.

Continued service on the Committee on Environmental Health of the Montana State Advisory Council on Comprehensive Health Planning.

Continued service on the Board of Directors of the Montana Division of the American Cancer Society.

Co-chairman Leptospirosis Committee of the U.S. Animal Health Assn.

Serial No. NIAID-171
1. Rocky Mountain Laboratory
2. Rickettsial Diseases Sect.
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Rickettsiae and rickettsial infections

Previous Serial Number: Same

Principal Investigators: W. Burgdorfer, R. A. Ormsbee, and R. L. Anacker

Other Investigators: L. P. Brinton, E. A. Casper, R. K. Gerloff, R. N. Philip, R. F. Smith, L. A. Thomas, and C. E. Yunker

Cooperating Units: Dr. T. R. Adkins, Dept. of Entomology, Clemson Univ., Clemson, South Carolina
Dr. P. Fiset, University of Maryland, School of Med., Baltimore
Mr. A. Hicks, Jefferson County Health Dept., Birmingham, Alabama
Dr. B. R. Norment, Dept. of Entomology, Mississippi State Univ., State College, Miss.
Dr. J. J. Plorde, Veterans Administration Hospital, Seattle
Dr. V. S. Padbidri, WHO Fellow, Virus Research Center, Poona, India
Dr. D. J. Sexton, EIS Medical Epidemiologist, State Board of Health, Jackson, Miss.
WHO, Geneva, Switzerland

Man Years	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	14 5/12			15 4/12
Professional:	4 10/12		11/12	5 9/12
Other:	9 7/12			9 7/12

Project Description:

Objectives:

1) Studies of ecology and epidemiology of rickettsioses in selected areas of the world including the United States, 2) development and improvement of serologic and microbiologic methods useful in research of rickettsial diseases, 3) development of improved diagnostic reagents and tests, 4) study of rickettsial growth and survival under experimental conditions, 5) study of phase change in Coxiella burneti, 6) search for chronic or recrudescent Q fever, and 7) study of immune response in animals and man to rickettsial agents.

Methods employed:

Standard methods applied to rickettsial research in the field and in the laboratory have been employed. In addition, a variety of immunologic, chromatographic, and electrophoretic techniques plus improved modifications of these, have been used in preparation of purified rickettsiae and in studies of immune responses.

Major findings:

1. In cooperation with Dr. T. R. Adkins, a study was initiated to investigate distribution of Rickettsia rickettsii in South Carolina. Ticks off man and animals were submitted to Clemson University for identification and to this laboratory for examination of rickettsial agents. Of 900 ticks (732 Dermacentor variabilis, 88 Amblyomma americanum, 80 Rhipicephalus sanguineus) examined, spotted fever group rickettsiae were isolated from 36 specimens, all D. variabilis. Highest infection rates were seen in ticks removed from raccoons (19%) and the lowest among those taken from dogs (3.6%). However, almost 8% of D. variabilis taken from dogs in Birmingham, Alabama, were infected. Of particular interest was the isolation of R. rickettsii from a practically engorged D. variabilis off a patient, who, however, remained asymptomatic and did not develop antibodies to spotted fever rickettsiae. The patient had no previous history of spotted fever or vaccination.

In addition to above cited 36 rickettsial isolations, 24 D. variabilis and 15 A. americanum were infected with rickettsia-like microorganisms nonpathogenic for test animals.

Dr. D. J. Sexton, EIS Medical Epidemiologist assigned by CDC to the Mississippi State Board of Health, requested help in a study on the epidemiology of spotted fever in that state. A total of 58 clinically diagnosed spotted fever cases were questioned about symptoms, tick bites, and contact (including contact with tick-infested dogs). Multiple sera were obtained from these cases and were tested for antibodies against spotted fever antigens by complement fixation (CF), microagglutination (MA),

and indirect fluorescent antibody tests. The results of these studies are reported in NIAID-182.

Of 123 dogs from northern and central Mississippi tested for antibodies to R. rickettsii by CF, 7 were anticomplementary, 41 (35.3%) had antibodies in titers of ≥ 1:8. In a control group of dogs from metropolitan Chicago, one only had a titer. When tested by MA, 86 of 120 (71.5%) had titers of ≥ 1:8; 10 of 20 dogs (50%) in the control group were also positive. The poor correlation between CF and MA test results in both Mississippi and Chicago dogs strongly suggests a lack of specificity of the MA test.

Of 129 D. variabilis, 884 R. sanguineus, and 4 A. americanum collected from Mississippi dogs, only one D. variabilis (0.8%) was infected with R. rickettsii. On the other hand, 167 R. sanguineus (18.8%) were found infected with a rickettsial agent related to the spotted fever group. Preliminary tests (fluorescent antibody staining, pathogenicity for meadow voles and guinea pigs, biologic behavior in chick embryos, and in various types of tissue cultures) suggest this agent to be different from R. rickettsii (see also NIAID-186).

2. Modifications of zonal centrifugation procedures for purification of spotted fever rickettsiae, mentioned in last year's report, have led to preparations of much higher purity. Yields of purified rickettsiae per gram of host tissue were as high as 0.07 mg for yolk sac and 5.2 mg for L cells, with about 1×10^{10} organisms per mg dry weight. Results of chemical analyses of 2 preparations of purified R. rickettsii from L cells and of 2 preparations from yolk sacs were similar: nitrogen content varied from 11.9 to 12.4%, phosphorus from 1.32 to 1.58%, total carbohydrate from 4.62 to 5.57%, lipid from 27.8 to 31.1%, and protein from 63.7 to 68.9%.

Vaccine doses varying from 0.06 to 0.28 µg of purified material reduced fever in guinea pigs challenged with 10^5 ID_{50} of R. rickettsii to half that produced in controls. This compared with 34.8 µg and 41.6 µg of commercial vaccine required to achieve the same degree of protection. About 4 times less vaccine was needed for the intraperitoneal route than for the subcutaneous route.

3. Zonal rotor-purified R. rickettsii has been evaluated to a limited degree in the CF, MA, and hemagglutination tests. In the CF test, when compared with antigens prepared according to RML's standard procedure and another obtained from CDC, all three gave similar titers against specific antisera. However, while the standard RML and the CDC antigens reacted with antisera to yolk sac, the zonal rotor preparation did not. The CDC antigen, in addition, cross reacted also with Q and typhus sera. In the MA test, the zonal rotor-purified rickettsiae were agglutinated by specific guinea pig sera, although the titers were generally lower than those recorded by CF. Promising results were obtained also with the hemagglutination test for which purified rickettsiae were solubilized with

sodium hydroxide and adsorbed to sheep red blood cells. In several instances, antibodies could be detected in guinea pig sera which were negative by CF. Such antibody levels at the time of challenge with virulent R. rickettsii could be correlated with protection.

4. During the past year, conditions for optimal development of mouse L cell spinner cultures were established to provide a system for massive cultivation of R. rickettsii and related organisms. Cell growth was superior in spinner medium prepared from liquid concentrates than in media from powder concentrates. The 100 ml rubber-stoppered Bellco flasks provided more efficient cell growth than did the larger 200 ml and 500 ml sizes. Bellco multi-stirrers emit considerable heat, hence it was necessary to modify each unit and obtain better ventilation in order to control temperatures within the cultures. Quantitation of cells, which, if cells are clumped, has been undependable with hemacytometers, has been achieved with a modified thrombocytocrit, making it possible to determine the volume of packed cells in 1 ml suspension following centrifugation.

Because the parent L cell line was found to be more susceptible to R. rickettsii than the variant line, L 929, the former has been used even though the latter is supposed to be more spinner-adapted. The time required for complete annihilation of a culture was shown to depend on the multiplicity of infection (MOI). In 3 experiments where MOI were 8, 17, and 24, only 5.7% of input cells were still alive on the 4th day. In contrast, where MOI were 0.24 and 0.024, the percentage of alive cells did not fall to 1% until the 11th day. Maximum infectious titers also varied with MOI, but infectious titer did not seem to be a reliable indicator of over-all production of rickettsiae.

5. Exposure of R. rickettsii to specific immune serum does not prevent the rickettsiae from subsequently invading chick embryo cells in monolayer culture where they multiply and cause formation of plaques. These findings are consistent with results recently reported by Dr. Wisseman for R. prowazeki.

6. Additional attempts to cause a shift of phase I to phase II in embryonated eggs with cloned phase I C. burneti failed. Similarly, it was not possible to cause a shift of phase II to phase I by guinea pig passage of cloned phase II organisms; the phase II C. burneti could not be passed in guinea pigs for more than 2 consecutive times.

7. Patients with typhus group or spotted fever group rickettsial diseases were found to exhibit a wide spectrum of immune responses to both spotted fever and typhus group antigens. This was demonstrated by MA as well as immunofluorescence tests. Patients with epidemic typhus fever, for instance, have been shown to respond with high titers of antibodies against R. canada and R. akari. It appears that the degree of cross reactions elicited in patients with rickettsial disease is much greater

than is the case with experimentally infected guinea pigs. These cross reactions occur primarily in agglutination and immunofluorescence tests. The CF test appears to be a much more specific test for identifying the specific etiologic agent.

8. WHO Reference Center function. As a WHO Reference Center, RML supplied on request to 13 domestic and foreign laboratories 11 antigen preparations, 6 live seeds of R. rickettsii, R. prowazekii, R. typhi, R. canada, and C. burneti, 5 antisera against R. rickettsii, R. prowazekii, and C. burneti, and 100 ml of C. burneti CAT antigen. We continued to participate in the WHO-sponsored project designed to standardize methods and reagents used in the diagnosis of rickettsial infections.

Significance to biomedical research and the program of the Institute:

The steadily increasing number of spotted fever cases, especially in the South Atlantic and East South Central states, where 427 of a total of 635 U.S.A. reported cases occurred during 1973, has prompted medical and public health authorities to seek assistance in studies pertaining to the occurrence and prevention of this disease. Our observations in South Carolina, Alabama, and Mississippi clearly show the high endemicity of spotted fever in these areas and the exposure of people in rural as well as urban environments. They also suggest that the ecology of R. rickettsii is far more complex than has been generally assumed; new rickettsia/tick associations have been discovered and appear to be involved in human disease.

Our cooperative studies with Dr. Adkins constitute a significant step forward in the prevention of spotted fever through education of the general public. Through public media the people of South Carolina were informed that spotted fever is not a "historical curiosity" but a serious current health problem. To this, the public responded with great interest and enthusiasm as indicated by the large number of ticks (900) received for hemolymph tests for rickettsiae.

Detection in and isolation from the brown dog tick, R. sanguineus, of a spotted fever group rickettsia different from previously described agents represents the first incrimination of this tick as a rickettsial vector in the United States. R. sanguineus is said to feed on dogs only (at least in this country) and rarely bites man. However, its significance as the source for human infections is indicated, particularly in Mississippi, where numerous spotted fever cases were not associated with tick bites. Contraction of the causative agent from crushed ticks during deticking of dogs appears likely.

Purification of R. rickettsii by zonal rotor centrifugation is an important step toward more effective diagnostic rickettsial reagents. Rickettsiae purified by this method are far more immunogenic than are

preparations used in commercially available vaccines, and, when used in the CF test, they do not react with sera against other rickettsiae or against egg antigens.

Mass production of R. rickettsii and possibly of other rickettsiae in mouse L cell spinner cultures appears now possible without having to go through the monolayer step.

The mechanism of phase change in C. burneti is still an obscure phenomenon. However, discovery that cloned phase I strains are more stable than the parent strains is of importance in the production of vaccines against Q fever and of reliable reagents for diagnostic tests.

The finding that specific antiserum against R. rickettsii does not prevent this organism from invading and multiplying in primary chick embryo fibroblasts has importance in understanding the persistence of rickettsial infections in immune persons, and suggests a possible mechanism for the long-term persistence of rickettsiae in animal hosts. It also suggests that the previously demonstrated potency of immune serum in passively transferred immunity depends upon the presence of specific phagocytic cells as well as humoral antibodies.

The degree of serologic cross reactions found within the typhus-spotted fever complex indicates that epidemiologic studies on specific rickettsial infections cannot be done reliably without the use of representative antigens from both major groups, and, at this point, the CF test. On the other hand, it suggests development of a universal reagent for the diagnosis of rickettsial disease caused by any member of the typhus and spotted fever groups. Although of limited usefulness in epidemiologic surveys, such a test may be of value in treatment of rickettsial disease patients.

The WHO-supported collaborative study on laboratory diagnosis of rickettsial infections will eventually lead to standardized serologic techniques and reagents.

Proposed course of project:

Guidance and support to outside agencies (health departments, universities, research laboratories, etc.) with emphasis on teaching investigators the recently developed techniques in rickettsiology, will continue to be a major function of RML's Rickettsial Diseases Section. Since education of the general public is the most important role in prevention of spotted fever, local, regional, and state health laboratories throughout the South Atlantic and East South Central states will be encouraged to initiate campaigns and tick surveys similar to those conducted in South Carolina. In addition, a workshop is presently being organized by the South Carolina State Health Department and by this laboratory to teach

technical personnel there the application of the hemolymph test as a dependable and rapid tool for detection of rickettsial agents in ticks taken off people or vegetation. Invitations for participation have been issued also to research centers and health laboratories in the neighboring states Florida, Georgia, Alabama, Tennessee, Kentucky, and North Carolina. If successful, similar workshops are anticipated in other parts of the country.

Characterization and identification of the newly detected spotted fever group rickettsia in R. sanguineus will be continued. In addition, a project is planned in cooperation with Dr. Norment, State College, Mississippi, to determine distribution and significance of R. sanguineus as a vector of rickettsial agents in Mississippi.

The role of Amblyomma ticks in the ecology of R. rickettsii will be the subject of an investigation with Dr. J. A. Hair, Oklahoma State University, Stillwater, Oklahoma.

Rickettsia-like, nonpathogenic microorganisms isolated from D. andersoni, D. variabilis, and A. americanum will be grown on tissue cultures and purified for studies of their relationship to pathogenic rickettsial agents.

Studies pertaining to the antigenic relationship between the spotted fever and typhus groups will be continued. Attempts will be made to isolate and identify antigenic components held in common, to identify the diagnostic and protective antigens, and to establish the nature of immune responses to them in animals and man.

Additional work is planned to perfect mouse L spinner cultures for mass production of rickettsiae. Rickettsial growth in tissue culture systems will be further investigated in an effort to clarify the complex relationship between host cell and intracellular parasite, and to find ways in which this may be modified or controlled.

Research will be continued on the mechanism of phase change in C. burneti, and attempts will be made to estimate the occurrence of chronic and recrudescent disease caused by this agent.

RML will continue to serve as a WHO Reference Center and to participate in the WHO-sponsored world-wide project on standardization of laboratory diagnosis of rickettsial infections.

Honors and Awards:

Dr. R. A. Ormsbee

Presented papers by invitation at the IX International Congress of Tropical Medicine and Malaria, Athens, Greece, and at seminars at the Institute of Virology, Slovak Academy of Sciences, Bratislava, Czechoslovakia, and the Department of Microbiology, University of Maryland School of Medicine, Baltimore.

Served as Co-Chairman of the Section on Rickettsial Disease at the IX International Congress of Tropical Medicine and Malaria, Athens.

Appointed Chairman pro tem of the Subcommittee on the Taxonomy of the Rickettsiales, International Committee on Systematic Bacteriology of the International Association of Microbiological Societies.

Awarded a Certificate of Appreciation by the Surgeon General, Dept. of the Army, for past service as a member of the Commission on Rickettsial Diseases.

Publications:

Tyeryar, F. J., Jr., Weiss, E., Millar, D. B., Bozeman, F. M., and Ormsbee, R. A.: DNA base composition of rickettsiae. Science 180: 415-417, 1973.

Burgdorfer, W.: Possible extra-human reservoirs of Rickettsia prowazeki: ticks. In Proceedings of the International Symposium on the Control of Lice and Louse-Borne Diseases. Washington, D.C., Pan American Health Organization, Scientific Publ. No. 263, 1973, pp. 109-113.

Ormsbee, R. A.: The hypothesis of extra-human reservoirs of Rickettsia prowazeki. In Proceedings of the International Symposium on the Control of Lice and Louse-Borne Diseases. Washington, D.C., Pan American Health Organization, Scientific Publ. No. 263, 1973, pp. 104-109.

Cooney, J. C. and Burgdorfer, W.: Zoonotic potential (Rocky Mountain spotted fever and tularemia) in the Tennessee Valley region. I. Ecologic studies of ticks infesting mammals in Land Between the Lakes. Am. J. Trop. Med. Hyg. 23: 99-108, 1974.

Burgdorfer, W., Cooney, J. C., and Thomas, L. A.: Zoonotic potential (Rocky Mountain spotted fever and tularemia) in the Tennessee Valley region. II. Prevalence of Rickettsia rickettsi and Francisella tularensis in mammals and ticks from Land Between the Lakes. Am. J. Trop. Med. Hyg. 23: 109-117, 1974.

Burgdorfer, W., Ormsbee, R. A., Schmidt, M. L., and Hoogstraal, H.: A search for the epidemic typhus agent in Ethiopian ticks. Bull. WHO 48: 563-569, 1973.

In press:

Burgdorfer, W.: Rickettsioses. (Introduction, Rocky Mountain spotted fever, Boutonneuse fever, North Asian tick typhus, Queensland tick typhus, Rickettsialpox, Murine (flea-borne) typhus fever, Scrub typhus, Q fever). In Hubbert, W. T., McCulloch, W. F., and Schnurrenberger, P. R. (Eds.): Diseases Transmitted from Animals to Man (ed. 6).

Burgdorfer, W.: Rickettsial Diseases. (Introduction, Epidemic (louse-borne) typhus, Murine (flea-borne) typhus, Rickettsialpox, Queensland tick typhus, Trench fever). In Hunter, G. W., Frye, W. W., and Swartzwelder, J. C. (Eds.): Manual of Tropical Medicine (ed. 5).

Ormsbee, R. A.: Rickettsiae. In Blain, J. E., Lennette, E. H., and Truant, J. P. (Eds.): Manual of Clinical Microbiology (ed. 2).

Ormsbee, R. A.: Rickettsioses. In Hunter, G. W., Frye, W. W., and Swartzwelder, J. C. (Eds.): Manual of Tropical Medicine (ed. 5). (Q fever (Revision), American spotted fevers, Related spotted fevers and rickettsioses).

Serial No. NIAID-173
1. Rocky Mountain Laboratory
2. Zoonoses Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Selected zoonoses of regional importance

Previous Serial Number: Same

Principal Investigator: J. F. Bell

Other Investigators: H. G. Stoenner

Cooperating Units: Dr. D. C. Blenden, Microbiology, University of Missouri, Columbia
Dr. H. Fred Clark, Wistar Institute, Philadelphia, Pa.
Fukushima Medical College, Fukushima, Japan:
Dr. Nobuo Kusonoki and Dr. Yo Yamagata
Dr. Steven Harris, Pathology, Veterans Administration Hospital, San Diego, Calif.
Dr. Fred Hochberg, Neuropathology, Massachusetts General Hospital, Boston
Dr. Robert Hoffman, Zoology, University of Kansas, Lawrence
Dr. Shoichiro Ohara, Director, Laboratory of Ohara General Hospital, Fukushima
Dr. D. P. Perl, Neuropathology, Brown University Medical School, Providence, R.I.

Man Years	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	3 10/12			3 10/12
Professional:	11/12			11/12
Other:	2 11/12			2 11/12

Project Description:

Objectives:

To determine the source and significance of contamination of streams by Francisella tularensis, the ecology of the rabies virus, the pathogenesis of rabies, and central nervous system (CNS) immunology.

Methods employed:

Field work in ecology and routine laboratory techniques of microbiology, entomology, and immunology are employed as necessary for surveillance of regional outbreaks of disease.

Major findings:

1. Tularemia. During epizootics of tularemia in microtine rodents in the Northern Hemisphere in both the Old World and New World, natural streams can be heavily contaminated with F. tularensis. Hence, there is inference that these rodents are responsible for contamination of water also during enzootic periods. However, the source of the protracted contamination so frequently seen cannot be attributed to carcasses of infected animals, and (except for one observation of bacteriuria in a naturally infected vole at this laboratory) infection of voles with virulent type B organisms causes only acute infections. We have produced chronic infections in beavers, voles, and mice by vaccination with attenuated live organisms and subsequent inoculation of virulent bacteria. We have also produced chronic bacteriuria, but only in voles. This is a completely artificial mechanism without a known counterpart in nature. But in recent experiments, chronic bacteriuria was produced by feeding increasing doses of virulent organisms, a means by which infection could occur in nature. We believe that this could account for contamination of streams in interepizootic periods.

The histopathology of chronic shedding tularemia pyelonephritis is being studied in collaboration with several Japanese pathologists. Renal infection is generally thought to result from ascending bacterial invasion. Our results are difficult to explain on the basis of a hematogenous route. Limitation thus far of chronic pyelonephritis to voles is puzzling.

2. Rabies.

a. Effects of a hyperthermic ambient upon the progression of rabies infection in mice are more complex than they first appeared to be. Contrary to published statements about other species, mice have lower-than-normal temperatures at onset of infection. High ambient temperatures that have relatively little effect on body temperatures of normal mice significantly raise the temperatures of infected mice. We had ascertained that reduction in mortality and increase in abortive infection were proportional to the total time infected mice were exposed to increased

ambient temperature. Recent observations show that delayed exposure to hyperthermia during the incubation period delays onset of illness.

b. Rabid bats (4) were found for the 20th consecutive year—probably a record. It is significant that no other species of animal has been found rabid in this area during that time.

A colony of Epesticus fuscus bats resident in the attic of a local hospital, from which a rabid bat had been obtained, was captured for observation and study. There was no evidence of carrier rabies or recovery from rabies infection in the remainder of the colony.

We are collaborating with Dr. R. Hoffman on the distribution of bats in Montana. Several species, some of which are common, have been reported for the first time in the state.

c. Investigation of efficacy of various rabies vaccines, both experimental and commercial, has revealed marked differences in mechanisms of action, effective routes of administration, undesirable side effects, and usefulness in primary and sequential application. The immunity elicited is enhanced by inoculation of different kinds of vaccines in primary and booster inoculations. Moreover, allergic reactions to successive inoculations are reduced.

d. The histopathology of spontaneous abortive rabies is unknown except for sporadic reports of individual cases. In collaboration with Dr. Hochberg, we are studying the progression of changes in the CNS from onset of infection to recovery.

Dr. Blenden and associates are studying the occurrence of rabies antigen (detected by FA) in the skin. Our techniques for producing abortive rabies have enabled them to extend their work to late stages of infection and recovery. Results thus far indicate that antigen disappears during recovery.

Clinical and immunologic observations have been continued on 5 dogs and one cat that survived rabies infection several years ago. One dog had minor sequelae but the cat severe sequelae. During repeated monitoring of antibody content in its serum and cerebrospinal fluid (CSF), we observed that titers increased when the condition of the cat progressively deteriorated. The cat was killed 3 years after inoculation because of hindlimb paralysis, fasciculation of thigh muscles, and general deterioration, and subjected to intensive study here and at Brown University by Dr. Perl. It appears that the cat had chronic progressive rabies of long duration.

Continued studies on CNS immunity have shown that not all forms of encephalitis result when CNS antibodies are present. Animals recovered from rabies (or with chronic rabies) maintain significant CSF titers for at least 4 years—the longest period tested. However, rabies antibodies

induced by vaccination with either live or inactivated vaccine do not appear in the CSF in significant titer, even when serum titers are very high. Serum antibodies to other antigens do not appear in the CSF in abortive rabies, and abortive Western equine encephalitis does not (in limited tests) give rise to antibodies in the CSF. In continued experiments, we have found that experimental allergic encephalitis (EAE) does not breach the barrier; antibodies in the serum do not appear in the CSF.

3. Relapsing fever. Borrelia hermsii has now been carried through 35 subcultures in Kelly's medium. As judged by generation time, maximum growth, and length and motility of the organism, the process of adaptation to growth in the medium extended through at least 11 passages. More than 800 organisms in mouse plasma were required to establish growth in this medium, but only a single organism of a fully adapted passage (25th) was required for a successful subculture.

Significance to biomedical research and the program of the Institute:

The experimental production of chronic (fatal) shedding tularemia nephritis in voles demonstrates the potential ability of infected voles to contaminate soil and water. A previous hypothesis about the source of contamination emphasized the role of mud as a growth medium in lotic waters. But F. tularensis fails to grow on excellent media at the temperatures of naturally contaminated streams. If voles are the principal or only source for contamination of waters, access of voles to rural domestic water supplies should be eliminated.

The ability of F. tularensis to cause chronic interstitial nephritis in voles, but not in mice or beavers, has not been explained. We hope that further inquiry into the fundamental differences in the responses of these species to this organism may lead to a better understanding of the pathogenesis of pyelonephritis and the genesis of hematogenous kidney lesions.

Inhibition of infection by several viruses in homiothermic animals and by rabies virus in bats has been recorded, but effects of a high thermic ambient on rabies infection has not previously been described. Our earlier studies that showed diminished mortality and increased abortive infection in proportion to time of exposure to high ambient temperature suggested direct effect of temperature upon virus. The subsequent observation that delayed exposure delays onset of infection reveals that inhibition is probably physiologic. Temperature at onset of infection is lowered rather than increased, as reported in the literature. Although temperature at onset can be raised significantly by prior and continued exposure to high ambient temperature (a reaction that increases survival), exposure to high ambient temperature after onset is not advantageous. Therefore, induced hyperpyrexia would not seem to be useful in therapy.

Continuous occurrence of rabies in bats for 20 years in this area and absence of known rabies in any other species reinforces the concept of compartmentalization of the disease. There is reason to believe that it is essentially a disease of one species, E. fuscus. The disease should be considered a continuous "natural" occurrence in a population (rather than an episodic catastrophe) that merits constant vigilance rather than hysterical reaction.

Observations on CNS antibodies, largely resulting from investigations in our laboratory for many years, are only beginning to be recognized and used in human medicine. These observations are of value in the differential diagnosis of postinfectious, postvaccinal and allergic encephalopathies, in the identification of abortive rabies infection in animals, and in tracing the course of chronic or recrudescent CNS rabies infection.

Persistence of rabies antibody in the CSF for years after CNS infection implies that antigenic stimulation is continuous. If antibodies are produced in situ, the persistence needs explanation. If antibodies are produced peripherally (unlikely) and continue to penetrate the blood/brain barrier, continued permeability of that barrier long after subsidence of infection is also puzzling.

Zlotnik and Grant (1973) showed marked differences in the histologic reaction of the CNS to IC challenge with virulent virus after vaccination with live and inactivated rabies vaccines. Our studies have shown the reaction of immunity in abortive rabies without prior immunization. There is increasing evidence that allergic reaction in CNS infection at times is responsible for severe damage to the CNS and for sudden death. About one of 600 cases of measles severely involves the CNS and results in distressing sequelae. Many other infectious agents (e.g., mumps, vaccinia, herpes, Coxsackie, arboviruses, poliovirus, Papovaviruses) also infect the CNS and cause dire immediate effects and severe sequelae. In view of the frequency and severity of these occurrences, it is astonishing that NIAID relegates this study to very low priority and gives it very poor support.

Cereghino states (and I agree) that the definitive diagnosis of rabies during life is difficult. Correlation of skin antigen with active infection could be useful in that regard. The source and significance of the antigen are not known.

EAE seems to be the equivalent of postrabies-vaccinal encephalitis, a too-frequent accompaniment to vaccination for rabies and too often confused with rabies encephalitis. Failure of EAE to breach the blood/brain barrier means that the two conditions can be differentiated by the finding of rabies antibodies in the CSF in abortive rabies, but not in postvaccinal encephalitis.

Proposed course of project:

The success in producing tularemic nephritis in voles with chronic shedding of F. tularensis in urine has been gratifying because it provides a possible explanation for the natural contamination of streams and because histopathologic study of the lesions is possible. Two manuscripts on these subjects are being prepared. The irregularity in producing nephritis, its limitation to voles, and the contamination of soil by urine will be subjects of continuing study.

Bat specimens suspected to be rabid will be examined without special attempts to investigate prevalence. A manuscript on the effects of primary and booster vaccinations is being prepared. The first phase of studies on hyperthermic ambient temperature has been completed. Work will be continued with temperature sensitive mutants. Histopathology of abortive and chronic rabies in mice will be discontinued when Dr. Hochberg completes his studies. (The manuscript is expected soon.) Studies of the chronic disease in dogs and cats will be continued with Dr. Dan Perl. Elucidation of the factors responsible for chronic rabies will be emphasized.

Work will be continued on the permeability of the blood/brain barrier to antibodies formed in the CNS and peripherally. The puzzling persistence of CSF antibodies long after apparent termination of CNS infection will be investigated further.

Honors and Awards:

Dr. J. F. Bell

Appointment to Governor's (Montana) Advisory Council on Rodents, Predators and Rabies Control.

Continued appointment as Faculty Affiliate, Zoology Department, University of Montana.

By invitation, gave talk on "Rabies" at University of Montana, March 7, 1973.

By invitation, gave talk on "Rabies" at Saint Patricks Hospital, Missoula, July 5, 1973.

Invited to contribute chapter on "Tularemia" in Handbook of Bacterial Infections in Animals, Gustav Fischer.

Publications:

Stoenner, H. G.: Application of serologic findings to the diagnosis of leptospirosis. In Proceedings 76th Annual Meeting of the U.S. Animal Health Association, Miami Beach, Florida, November 6-9, 1972. Richmond, Va., U.S. Animal Health Association, 1973, pp. 622-634.

In press:

Bell, J. F. and Munoz, J. J.: Enhancement of immune response to rabies virus by Bordetella pertussis extract. Proc. Symposium sur la Rage, Lyon, France, 1972.

Bell, J. F.: Latency - abortive rabies. In Baer, G. M. (Ed.): The Natural History of Rabies. New York, Academic Press.

Serial No. NIAID-174
1. Rocky Mountain Laboratory
2. Medical Entomology and Acarology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Systematics and vector relationships of certain parasitic arthropods

Previous Serial Number: Same

Principal Investigator: J. M. Brennan

Other Investigators: C. M. Clifford, J. E. Keirans, C. E. Yunker, and B. N. Chaniotis

Cooperating Units: *Miss Mona Al Taqi, Univ. of Kuwait, Kuwait
Dr. Omar M. Amin, University of Wisconsin-Parkside, Kenosha
Brigham Young University, Provo, Utah: Mr. J. T. Reed, Mr. R. Saunders, Dr. Selby Herrin
*Dr. A. Canaris, Univ. of Texas, El Paso
Mrs. M. B. Chitwood, Primate Research Center, Univ. of Calif., Davis
Dr. J. A. Hair, Oklahoma State Univ., Stillwater
Dr. T. M. Hefnawy, Cairo University, Cairo, A.R.E.
Dr. R. E. Hopkins, Travenol Laboratories, Morton Grove, Ill.
Dr. K. M. Howell, Univ. of Dar es Salaam, Tanzania
*Dr. Murray Johnson, Univ. Puget Sound, Tacoma, Wash.
Dr. R. B. Loomis, Calif. State Univ., Long Beach
Dr. W. P. Melaney, ARS, USDA, Albuquerque, N.M.
NAMRU-3, Cairo, A.R.E.: *Dr. H. Hoogstraal and Dr. M. N. Kaiser
Mr. R. O'Donnell, Univ. Mont., Missoula
*Dr. T. A. Okereke, Univ. Benin, Nigeria
Pacific Research Station (NIAID), Honolulu, Hawaii: Dr. Leon Rosen and Dr. D. Gubler
Mr. James Reddell, Texas Tech University, Lubbock
Dr. Jon P. Shoemaker, VA Hospital, Huntington, W. Va.
Dr. D. Sonenshine, Old Dominion Univ., Norfolk, Va.
Dr. D. E. Worley, Vet. Res. Lab., Montana State Univ., Bozeman

* = material identified in large number or on a continuing basis.

Man Years	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	7 2/12			7 2/12
Professional:	2 10/12			2 10/12
Other:	4 4/12			4 4/12

Contract Support:

Contract Number	Project Officer (NIAID)	Contractor	Contractor's Project Director	Annual Funding Level
PL-480 03-005-1	C. M. Clifford J. E. Keirans H. G. Stoenner	NAMRU-3 Cairo, Egypt	H. Hoogstraal	$60,360

Project Description:

Objectives:

Taxonomy, biology, and colonization of parasitic arthropods of known and potential medical importance.

Methods employed:

Standard, adapted, and conceived techniques for collecting, handling, rearing and colonizing parasitic arthropods and their preparation for taxonomic study. Collaboration with appropriate sections at RML and other agencies and institutions. Identification and clarification of systematic problems, principally of ticks and parasitic mites.

Major findings:

1. Ticks (Clifford and Keirans). An extensive manuscript on the species of Ixodes from Nepal has been completed. Previous to this work only 2 species were known from Nepal. We now recognize 4 subgenera and at least 15 species.

A species of a remarkable new genus of Argasidae has been collected in a bat cave in Campeche, Mexico, and is presently being described with the aid of the scanning electron microscope (SEM). This will be the first new tick genus described with SEM photos supplementing line drawings.

Two new subgenera of Ixodes and 2 new species of Amblyomma were described, and identifications were made for various individuals and organizations as requested.

Because of the transfer of Dr. Hoogstraal's extensive library and tick collection, space adjustments are being made in the library for a special section to house the Hoogstraal collection.

PL-480 project 03-005-1 included an on-site visit to NAMRU-3 by Dr. Keirans.

Scanning electron microscopy. An intensive study has been initiated on African species of the economically important genus Rhipicephalus. This taxonomically difficult genus contains at least 40 species, several of which transmit a variety of pathogenic organisms to cattle, horses, sheep, and other livestock. With the SEM we have detailed specific characters, not seen by classical methods, within species-complexes such as the sanguineus group.

Computerization of tick data. The plans to computerize the data concerning the RML and NAMRU-3 tick collections are formalized and we are hopeful that a contract will be awarded this spring. The final agreement is to award the contract locally and have the work done at the RML. This will save the government several thousands of dollars and, in our opinion, will result in a better effort for all parties concerned.

2. Parasitic mites (Yunker). Methodology for SEM visualization of parasitic mites is being developed. These arachnids, unlike ticks, are soft-bodied and readily collapse upon prolonged exposure to vacuum. Infiltration of specimens with low molecular weight plastic resins, followed by partial polymerization of the resin and its removal from the exterior of the specimen by solvent washes have yielded promising initial results. Further tests are in progress.

The project entitled Biochemical taxonomy of ticks (see last year's report) continues to languish due to lack of insectary support.

A review of South American species of the large parasitic mite genus of Hirstionyssus was completed and is in press.

3. Susceptibility of Aedes (Stegomyia) mosquitoes to dengue virus (Chaniotis). The objective of this and related studies is to produce a mosquito strain refractory to dengue viruses and able to compete favorably under natural conditions. At RML work has focused on testing susceptibility of Aedes aegypti (Niue strain), Ae. albopictus (Oahm strain), and Ae. polynesiensis (Samoan strain) to dengue 1. The 3 species of mosquitoes are fed simultaneously undiluted, 1:10, and 1:100 virus suspensions combining

of equal parts of virus, erythrocytes (guinea pig) and 10% sucrose solution. After an extrinsic incubation period of 21 days at 82° F the mosquitoes are harvested, frozen at -70° C and shipped to PRS in Honolulu where the frequency of infection and virus titers are determined by direct fluorescent antibody technique, and plaque assay in tissue culture. Major findings and significance of the project to biomedical research are to be reported by Dr. Leon Rosen in his annual report. Proposed course of the project awaits assessment of the results of the current experiments.

4. Chiggers (Brennan). A rather large review of Venezuelan chiggers (135 species in 38 genera) is completed and in press as is a study of the medically important genus Eutrombicula (22 species) of the Neotropical fauna. Relative to chigger systematics, 4 manuscripts have been published and 2 are in press.

Three new genera and 11 new species of Neotropical chiggers were described, and identifications were provided as requested. A study of Surinam chiggers is being continued.

5. Colonies of medically important arthropods. In 1973 the RML insectary provided 1,199 ticks of 6 species for research needs at RML and elsewhere. This figure is down from 155,890 specimens provided in 1972. This very drastic reduction in numbers clearly reveals the need for a full-time insectary attendant. The research goals among staff members at RML and at other research facilities using parasitic arthropods supplied by our insectary are being seriously impeded because of this unfortunate personnel policy.

Currently, 29 tick, 2 mite, and 4 mosquito species are under colonization.

Significance to biomedical research and the program of the Institute:

As in the past, where arthropods are concerned in human disease, their systematic studies are a requisite to all research issuing therefrom. The taxonomically and biologically trained personnel of this section, in addition to providing numerous identifications, are frequently consulted for information involving protection from and control of medically important arthropods, and are even appealed to for aid in solving problems of entomophobia. Over and above basic research, various services are provided. The arthropod colonies are used in arthropod tissue culture, transmission studies, establishing subcolonies, teaching rearing techniques, etc.

Proposed course of project:

Continued investigations of parasitic arthropod systematics and biology, and provision of services, with emphasis on ticks, mites, and mosquitoes.

Publications:

Clifford, C. M., Sonenshine, D. E., Keirans, J. E., and Kohls, G. M.: Systematics of the subfamily Ixodinae (Acarina: Ixodidae). I. The subgenera of Ixodes. Ann. Entomol. Soc. Am. 66: 489-500, 1973.

Keirans, J. E., Hoogstraal, H., and Clifford, C. M.: The Amblyomma (Acarina: Ixodidae) parasitic on giant tortoises (Reptilia: Testudinidae) of the Galápagos Islands. Ann. Entomol. Soc. Am. 66: 673-688, 1973.

Hoogstraal, H., Clifford, C. M., Saito, Y., and Keirans, J. E.: Ixodes (Partipalpiger) ovatus Neumann, subgen. nov.: identity, hosts, ecology, and distribution (Ixodoidea: Ixodidae). J. Med. Entomol. 10: 157-164, 1973.

Yunker, C. E.: Parasites of endothermal laboratory animals. Chapter 15. Mites. In Flynn, R. J. (Ed.): Parasites of Laboratory Animals. Ames, Iowa, Iowa State Univ. Press, 1973, pp. 425-492.

Brennan, J. M. and Reed, J. T.: The Neotropical genus Aitkenius: three new species and other records from Venezuela (Acarina: Trombiculidae). J. Parasitol. 59: 531-535, 1973.

Hoogstraal, H., Clifford, C. M., and Keirans, J. E.: Argas (Microargas) transversus (Ixodoidea: Argasidae) of Galápagos giant tortoises: description of the female and nymph. Ann. Entomol. Soc. Am. 66: 727-732, 1973.

Herrin, C. S. and Yunker, C. E.: A new species of Hirstionyssus from Liomys irroratus in Texas (Acari: Mesostigmata). J. Med. Entomol. 10: 269-273, 1973.

Keirans, J. E.: Ixodes (I.) montoyanus Cooley (Acarina: Ixodidae): first description of the male and immature stages, with records from deer in Colombia and Venezuela. J. Med. Entomol. 10: 249-254, 1973.

Brennan, J. M. and Reed, J. T.: More new genera and species of chiggers (Acarina: Trombiculidae) from Venezuela. J. Parasitol. 59: 706-710, 1973.

Yunker, C. E. and Chitwood, M. B.: Note on the occurrence of larval filariae in a mite parasitic on bats. Acarologia 14: 530-532, 1972.

Yunker, C. E. and Saunders, R. C.: Venezuelan Macronyssidae. I. The genera Acanthonyssus Yunker and Radovsky, and Argitis, n. gen. J. Med. Entomol. 10: 371-381, 1973.

Saunders, R. C. and Yunker, C. E.: Venezuelan Macronyssidae. II. Nycteronyssus desmodus, n. gen., n. sp., off a vampire bat. J. Med. Entomol. 10: 381-384, 1973.

Brennan, J. M. and van Bronswijk, J.E.M.H.: Endoparasitic chiggers. VII. A redescription of Kymocta Yunker and Brennan, 1962, a new species from Venezuela, and other records (Acarina: Trombiculidae). J. Med. Entomol. 10: 449-451, 1973.

Sonenshine, D. E. and Clifford, C. M.: Contrasting incidence of Rocky Mountain spotted fever in ticks infesting wild birds in eastern U.S. Piedmont and coastal areas, with notes on the ecology of ticks. J. Med. Entomol. 10: 497-502, 1973.

Keirans, J. E., Clifford, C. M., and Radovsky, F. J.: Argas (Argas) monachus, new species (Ixodoidea: Argasidae), from nests of the Monk Parakeet, Myiopsitta monachus, in Argentina. J. Med. Entomol. 10: 511-516, 1973.

Brennan, J. M. and Reed, J. T.: Endoparasitic chiggers: VIII. The intradermal genus Intercutestrix Brennan and Yunker, 1966, and three new species (Acarina: Trombiculidae). J. Parasitol. 60: 185-187, 1974.

Chaniotis, B. N.: Sugar-feeding behavior of Lutzomyia trapidoi under experimental conditions. J. Med. Entomol. 11: 73-79, 1974.

Chaniotis, B. N.: Comparative flying and biting activity of Panamanian phlebotomine sandflies in a mature forest and adjacent open space. J. Med. Entomol. 11: 115-116, 1974.

In press:

Bain, O. and Chaniotis, B. N.: Befilaria puertoricensis n. sp. nouvelle Filaire Oswaldofilariinae d'Iguanidae aux Caraibes (Puerto Rico). Bull. Mus. Nat. Hist.

Brennan, J. M. and Reed, J. T.: A list of Venezuelan chiggers, particularly of small mammalian hosts (Acarina: Trombiculidae). Brigham Young Univ. Sci. Bull.

Keirans, J. E. and Clifford, C. M.: Ixodes (Pholeoixodes) conepati Cooley and Kohls (Acarina: Ixodidae): description of the immature stages from rock squirrels in Texas. J. Med. Entomol.

Stark, H. E., Inlao, I., Clifford, C. M., Keirans, J. E. and Lakshana, P.: A preliminary checklist of the ticks of Thailand. Appl. Sci. Res. Corp. J. Thailand.

Keirans, J. E.: The Rocky Mountain wood tick and the American dog tick. Chapter in Great Plains Range and Livestock Management.

Herrin, C. W. and Yunker, C. E.: Systematics of Neotropical Hirstionyssus mites, with special emphasis on Venezuela (Acarina: Mesostigmata). Brigham Young Univ. Sci. Bull.

Saunders, R. C. and Yunker, C. E.: Venezuelan Macronyssidae. IV. A new genus and species of rodent-parasitizing Ornithonyssinae (Acarina, Macronyssidae). J. Med. Entomol.

Serial No. NIAID-175
1. Rocky Mountain Laboratory
2. Allergy and Immunology
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Allergy, its mechanisms and role in disease

Previous Serial Number: Same

Principal Investigators: J. J. Munoz, B. W. Chesebro, J. E. Coe, D. L. Lodmell, and R. K. Bergman

Other Investigators: M. E. Bloom, W. J. Hadlow, T. A. Jackson, J. L. Portis, and R. E. Race

Cooperating Units: Dr. George Diamandopoulos, Dept. of Pathology, Harvard Medical School, Boston, Mass.
Dr. Ira Green, NIAID, NIH
Dr. A. L. Notkins, Chief, Laboratory of Oral Med., NIH
Dr. Jack Stimpfling, McLaughlin Research Institute, Great Falls, Montana

Man Years	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	16 6/12			16 9/12
Professional:	6 1/12		3/12	6 4/12
Other:	10 5/12			10 5/12

Project Description:

Objectives:

1) To study fundamental bases of hypersensitivity and its role in disease, 2) to investigate the mechanisms of slow virus infections, 3) to find methods of demonstrating these viruses and to develop diagnostic immunological methods, 4) to learn more about the function of immunoglobulin classes and the factors that affect their production, and 5) to study specific and nonspecific mechanism of virus and tumor immunity.

Methods employed:

Typical immunological, biochemical, biophysical, and serological techniques are used. Fluorescent and isotope labelling of antibodies, electron microscopy, radioimmunoelectrophoresis, and tissue culture techniques are widely used. Hamsters, inbred mice, mink, goats, sheep, guinea pigs, and rabbits are employed in various aspects of this project.

Major findings:

1. Studies on cell-mediated immunity in virus infection (Lodmell).

a. In vitro studies of cell-mediated immunity in virus infections. A recently developed in vitro assay for determining the effects of leukocytes on virus-infected cells has been used to determine if specifically sensitized leukocytes are more effective than normal unsensitized leukocytes in inhibiting herpes simplex virus (HSV - type I) replication. Peritoneal exudate leukocytes obtained from rabbits that had been injected with complete Freund's adjuvant (CFA) were "activated" by incubation with tuberculin (PPD) and added to monolayers of rabbit kidney cells that had been infected with HSV. Monolayers incubated with CFA-sensitized leukocytes and PPD contained 90% to 99% less virus than monolayers incubated with CFA-sensitized leukocytes without PPD. The inhibition of HSV replication by activated CFA-sensitized leukocytes was found to be dependent upon several factors, including the sensitivity of the leukocytes to PPD as reflected by the magnitude of skin tests in rabbits, concentration of PPD, and the number of leukocytes. Under optimal conditions, a leukocyte-to-target cell ratio of only 0.1 reduced the virus titer by over 97%. Also, activated splenic leukocytes were much less efficient in the inhibition of viral replication than cells from the peritoneal exudate. Supernatant fluids resulting from the interaction of PPD with CFA-sensitized leukocytes markedly inhibited HSV replication. Interferon was identified in these supernatant fluids.

Additional studies showed that leukocytes from rabbits specifically immunized with HSV can exert these same effects after activation with ultraviolet-light-inactivated HSV antigen. As in the case of PPD, supernatant fluids from the stimulated HSV-sensitized leukocytes also contained interferon.

b. In vivo studies of host containment and cure of virus infections. An in vivo assay is being used to determine ways to protect the host against skin lesions and genital infections by herpes simplex (types I and II) and vaccinia viruses. Preliminary studies have focused on presence of various lymphokines and accumulation of activated leukocytes at the sites of infection as induced by specific and nonspecific inflammatory reactions.

c. Genetic control of the immune response in determining resistance to herpes simplex virus infection in different murine strains. In collaboration with Dr. Chesebro, the intriguing observation that different murine strains vary markedly in resistance to herpes simplex virus infection is being investigated. In preliminary results, 3 murine strains were found to be highly susceptible to infection, and 2 strains were markedly resistant. With this background material we are attempting to discern if susceptibility to infection is genetically determined, if it is linked to the major histocompatibility locus, and if it may possibly be a multigenic trait.

2. Studies on Aleutian disease virus (ADV) (Bloom and Hadlow).

a. Assay of antibodies to ADV and antibody response in mink. The counter immunoelectrophoresis (CIEP) assay for ADV has been perfected and used extensively in the study of Aleutian disease (AD). The relationship of antibody response to disease, the relationship of genotype to antibody response and disease, and the specificity of the reaction are currently under study. The precipitating antigen has been found to be associated with the intact virion in precipitin lines examined by electron microscopy (EM).

In sapphire mink inoculated with the Pullman virus isolate, antibody response seems tantamount to subsequent disease, although with high dilutions of virus a lag period of up to 6 weeks has been noted. In pastel mink inoculated with Pullman virus, antibody formation occurs in the absence of overt disease or changes in the serum gamma globulin. The antibody response to parallel inocula of Pullman virus is significantly higher in sapphire mink than in pastel mink, and there is evidence that the antibody response in pastels may be transient.

The specificity of the reaction in CIEP has been well-documented in normal mink, mink ill with other natural diseases, and mink inoculated with Keyhole limpet hemocyanin and goat red blood cells (rbc). In addition, 18 laboratory workers with high exposure histories have been tested and found to be seronegative.

All of the above experiments have been done with antigen prepared from the Porter isolate of ADV. Plans include an evaluation of the antibody response to Porter ADV and further investigation of genetic influences on the immune response to the virus. Attempts to prepare viral CIEP antigen from Pullman virus materials have so far been unsuccessful. However, if this can be done, attempts to demonstrate antigenic differences between the 2 isolates have been planned.

3. Antibody response in in vitro spleen cultures of AD-affected animals (Bloom and Chesebro). Using spleen cell cultures from infected animals and ^{14}C-labelled medium, specific antibody formation to ADV in vitro has been demonstrated by using combined autoradiography and CIEP.

Experiments are in progress to utilize this technique to produce a primary immune response *in vitro* to the virus and subsequently to analyze the role of various cell populations in this response.

4. Studies on progressive pneumonia virus (PPV) (Bloom and Jackson). Buffy coats from 5 goats inoculated with PPV and 3 controls were examined in the EM. Virus-like particles (VLP) were seen in polymorphonuclear leukocytes and lymphocytes from all 8 goats. The VLP resembled PPV, visna virus and other members of the oncornavirus family. *In vitro* incubation of buffy coats with mitogens did not affect the frequency of VLP. Results of studies for mycoplasmal contamination were negative.

Suspensions of lung from animals infected with PPV have been run over sucrose gradients in attempts to purify PPV. Using EM and negative staining, particles of virus have been seen at density 1.16 to 1.18. It is planned to develop a reverse transcriptase assay for PPV and to utilize it both as a tool in virus purification and also as an assay for PPV in tissue culture and infected animals.

5. Genetic mapping and mechanism of action of an H-2-linked host gene involved in facilitating recovery from Friend virus leukemia in mice (Chesebro). Analysis of mice with recombinations within the H-2 region indicates that the H-2-linked gene (RPV-1) influencing recovery from Friend's leukemia maps to the right of the present boundary of the known IR region alleles. Further crossovers are being studied at present to locate more accurately the position of the RFV-1 gene and to determine if it is different from the RGV-1 gene that influences resistance to Gross virus leukemia.

Studies of the antiviral humoral response and viral proliferation in congenic mouse strains differing only at the H-2 complex have not explained the differences in recovery rates from leukemia in one of these strains of mice. Assays are being developed to investigate the host antiviral and antitumor cellular immune response and its role in recovery from this disease.

6. Studies on T and B cells in Syrian hamsters (Coe and Portis). The lymphocytes of man and mice have been found to consist of at least 2 populations, distinguished by markers on the surface of their membranes. For instance, B cells (Bursal equivalent, antibody precursor cells) have easily detectable (i.e., by FA technique) membrane bound immunoglobulin (Ig) and also a receptor which binds to complement (C_3 receptor). T and B cell populations have been defined using these 2 markers in various lymphoid tissues of the Syrian hamster. Thirty percent of lymphocytes in lymph nodes and 50% of lymphocytes in spleen have FA detectable Ig light chains and the majority of these (i.e., 60%) also have C_3 receptors. Most of the membrane bound Ig appears to be either γM (40% to 60%) or γA

(10% to 40%) whereas γ_2 (5% to 20%) and γ_1 (<4%) are found least frequently. Further work will analyze the functional significance of these T and B cells in hamster tumor immune models.

In collaboration with Drs. Ira Green and George Diamandopoulos, two SV40 induced hamster leukemias have been characterized as to their T or B cell origin. The lymphocytes from both tumors had only $7S\gamma_2$ globulin on their surface and did not contain a complement receptor. Thus, these tumors originated from B cells. In addition, one of the tumors synthesized $7S\gamma_2$ protein which was detected in the serum. This immunoglobulin appears to be of monoclonal protein because of its restricted electrophoretic heterogeneity and also because it appears to have "idiotypic" specificity—i.e., rabbit antisera to the isolated Ig were specific for this particular protein after absorption with normal hamster serum or Ig. Preliminary observations indicate that this "idiotypic" antigen is also present on the membrane of the tumor cell, which suggests the identity of secreted and membrane bound Ig. Future work will analyze the possible relationship between the membrane bound Ig and a receptor specific for the SV40 virus.

7. Studies on immune system of mink and sheep (Coe and Portis). T and B cell populations have also been defined in mink and sheep, and about 50% to 60% of the Ig positive B cells of mink have a receptor for C_3. Because mink with AD display a distinctly deranged immune system, a quantitative and qualitative study of the distribution of B lymphoctyes in both lymphoid organs and in areas of cellular infiltration may provide clues as to the nature of the abnormality. Indeed, paravascular lymphocytic infiltrates in sections of kidneys from AD-affected mink bound complement, suggesting that B cells are a prominent component of these infiltrates. In addition, diseased glomeruli selectively bound complement. The nature and specificity of this phenomenon is being studied.

Characteristically, serum gamma globulin increases in the AD-affected mink. Because sapphire mink are more susceptible to AD virus than are pastels, a quantitative study was made of changes in serum γG, γA, γM and β_1 C induced by the disease. Normal sapphires had two times more γM and γG and more β_1 C than pastels. In AD-affected mink, γM remained unchanged, γA increased tremendously (714%) in pastels and smaller increases in γG were seen in both color phases. In contrast, β_1 C content declined in sapphires but increased 203% in pastels.

These data indicate that γA is an important Ig in the immunology of AD (showing the greatest relative increase) but only in sapphires. Future studies will examine the relationship between Ig response, immune complex composition and pathophysiology of the disease.

8. Immune response in the hamster (Coe and Portis). The hamster responds to immunization with hen egg albumin (HEA) in CFA by producing both γ_1 and γ_2 antibody, whereas HEA in saline evokes only a $7S\gamma_1$ response,

with $7S\gamma_2$ tolerance. Quantitative studies of this phenomenon are in progress using the Jerne hemolytic plaque assay. Preliminary results suggest that the $7S\gamma_1$ response is approximately 10-fold greater in animals immunized with HEA in incomplete Freund's adjuvant than with HEA in CFA. In this situation there is a direct quantitative relationship between the magnitude of the early γM response and the $7S\gamma_1$ response. It is of interest that $7S\gamma_2$ antibody producing cells are detected early in the immune response in animals given HEA in saline, a regimen which eventually evokes $7S\gamma_2$ tolerance.

Other efforts to investigate this phenomenon involve the role of the macrophage, which plays an important role in antigen presentation to T and B lymphocytes. Cytophilic antibodies and antigen binding properties of hamster peritoneal macrophages are being studied using the sheep rbc rosette technique. In contrast to other rodents, cytophilic antibody in hamsters has been identified as both $7S\gamma_1$ and γ_2 Ig. However, the proportion of the hemagglutinating antibody which is cytophilic for macrophages is dependent on the nature of the antigenic challenge. For instance, sheep rbc stromata in CFA evokes abundant cytophilic antibody. Yet, in animals immunized with whole sheep rbc, a comparable amount of hemagglutinating antibody is not cytophilic.

9. Secretory Ig of lower vertebrates (Coe and Portis). Comparison of the amino acid sequences of human alpha and mμ chains suggest that divergence of IgA and IgM took place about 175 million years ago, about the time of the emergence of reptiles. Studies of secretions of the bullfrog Rana catesbeiana, garter snake Thamnophis ordinoides, and turtle Chrysemys picta indicate that a 19 to 21S γM fulfills the role of secretory Ig in these lower vertebrates. That is, γM antigenically indistinguishable from serum γM is the major IgM in succus entericus and the only Ig in bile of these species. This finding is of interest, because mammalian γM is not found in these secretions. Future studies will evaluate the presence at this phylogenetic level of a specialized transport mechanism for this secretory IgM.

Significance to biomedical research and the program of the Institute:

The emphasis of our research has been on the immunologic aspects of certain viral diseases, with special efforts directed to developing specific tests for antibody to AD and on methods to demonstrate this virus in tissues. The progress made along these lines will facilitate further investigations on the pathogenesis of AD. Studies on the in vitro destruction of herpes virus by "activated" leukocytes indicate the importance of these activated cells on certain virus infections and has opened avenues of further investigation which should prove interesting.

The studies on kinetic aspect of resistance to virus infection and cancer are of importance in understanding factors involved in tumor immunity and resistance to viruses. The studies on virus leukemias also offer information on the role of immune competent cells in cancer immunology.

The studies of the distribution of T and B cells in different color phases of mink may give some insight into the marked differences in their susceptibility to AD, as well as on the role that each type of cell may play in this "immunologic disease". The marked increase in γA in sapphire mink after infection with AD may point to significant differences in susceptibility to AD.

Proposed course of project:

The work on chronic virus diseases and in vitro models to study various immunological aspects of the viral diseases will be continued. The genetic aspects of tumor resistance in Friend's virus leukemia will be continued and further studies on immunoglobulins and distribution and role of B and T cells in AD and in hamster leukemias will be continued. Immunological studies on chronic pneumonitis will be intensified.

Honors and Awards:

Dr. D. L. Lodmell

Invited to participate in the Ninth Gustav Stern Symposium on Perspectives in Virology, New York City, February 1974.

Invited to participate and present paper in a Herpesvirus Workshop sponsored by the Research Resources Branch of NIAID, NIH, Bethesda, Maryland, February 1974.

Invited to participate in a Workshop on the "Immunopathology of Virus Infections" held at the Annual Meeting of the American Association of Immunologists, Atlantic City, April 1974.

Dr. J. J. Munoz

Faculty Affiliate, University of Montana, Missoula.

Serves as member of the Council for Mid-Winter Immunologists Conference.

Dr. J. E. Coe

Faculty Affiliate, University of Montana, Missoula.

Publications:

Munoz, J., Bergman, R. K., and Jackson, T. A.: Hypersensitivity reactions to a methylated bovine serum albumin and human serum albumin in the skin and footpad of mice. Z. Immunitaetsforsch. 146: 443-453, 1974.

In press:

Munoz, J., Lodmell, D. L., Race, R. E., Jackson, T. A., and Hadlow, W. J.: Delayed hypersensitivity in normal and Aleutian disease affected mink. Z. Immunitaetsforsch.

Serial No. NIAID-177
1. Rocky Mountain Laboratory
2. Molecular Biology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Immune prophylaxis of mycobacterial infections

Previous Serial Number: Same

Principal Investigators: E. Ribi and D. L. Granger

Other Investigators: R. F. Smith

Cooperating Units: Dr. I. Azuma, Osaka University, Osaka, Japan
Dr. W. R. Barclay, AMA, Chicago
Dr. W. Brehmer, Robert Koch Institute, Berlin, Germany
Dr. T. J. Meyer, University of Colorado Medical Center, Denver
Dr. R. Parker, Hamilton Biochemical Research Lab., Hamilton
Drs. S. Salvin and J. S. Youngner, University of Pittsburgh, Pittsburgh, Pa.
Mr. S. M. Strain, Montana Lung Association Fellow, Hamilton

Man Years	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	8 7/12			8 7/12
Professional:	1 6/12			1 6/12
Other:	7 1/12			7 1/12

Project Description:

Objectives:

To develop an effective antituberculosis vaccine from purified fractions of mycobacteria, to study the nature of the host responses to these fractions, and to determine the relationship of delayed hypersensitivity to immunity.

Methods employed:

A variety of physical, chemical, and immunologic techniques was used to prepare and characterize cell walls of several mycobacterial species, strains and fractions derived from them, and to assay the response of experimental animals to these fractions. Protective potency of these preparations was determined in mice challenged by aerosol containing Mycobacterium tuberculosis H37Rv. Histologic examination of various organs from these mice was carried out.

Major findings:

1. Improved procedure for isolating P3. P3 is a high molecular weight trehalose ester of mycolic acids, and one of many lipids extractable with neutral organic solvents from the cell wall of the tubercle bacillus (see scheme of fractionation in NIAID-179). These lipids also contain the widely studied Wax D and cord factor which in turn contain P3 in yields of about 15% and 50%, respectively. Cord factor is conventionally prepared from Wax C which itself contains only a limited quantity of P3 (about 1% or less). During a search for a more productive procedure to meet the increasing demands for P3, we found that the chloroform-methanol (CM) 2:1 soluble portion of Wax D, which is readily available in large quantity, is a far superior source. This extract was further fractionated by serial elution with CM mixtures of increasing polarity on columns of silicic acid and cellulose/DEAE (free base form) to provide yields of 95% pure P3. Because this product does not contain P2 but merely P4 and P1 as contaminants (see NIAID-179) it is purified by pressure-elution chromatography through microparticulate silica using CM 9:1 as the mobile phase. This final step can be completed in a short time since solvent changes are not needed.

2. Composition and structure of P3. P3 that we recover from M. bovis strain BCG, M. tuberculosis, strain Aoyama B, and M. kansasii is undoubtedly the active principle of the cord factor originally described by Noll and Bloch. It can be split by saponification into the sugar, trehalose, and a mixture of at least 3 different 90-carbon mycolic acids. The mass spectrum of only one resembled that of a known mycolic acid (α-mycolic acid), but those of the other 2 acids are not yet known. If these mycolic acid residues are esterified to trehalose in 2 positions (6 and 6'), we must expect a mixture of several structurally different mycolates. Therefore, P3 would not be expected to be homogeneous, but chromatographic methods presently available are not sensitive enough to resolve mixtures consisting of trehalose-dimycolates. Of additional interest is the fact that the mycolic acid methyl ester fractions prepared from the cell wall skeleton (CWS) of M. tuberculosis Aoyama B or M. kansasii and chloroform-methanol insoluble (CMI) fragments from CWS gave the same multiple-band chromatographic pattern as the mixture isolated from P3 (see NIAID-179). The mass spectra of separated components were identical with those obtained from P3. This finding is important in the correlation of biologic activities with structure of CWS and CMI.

3. Components of mycobacteria involved in protection and granuloma formation in mice. We reported last year that 2 ingredients in CW vaccines were needed for protecting mice against airborne challenge with H37Rv and for maximum production of granulomata in the lungs of mice. One is certainly P3. However, evidence is accumulating that rather than the peptidoglycolipid CWS or CMI per se, the other ingredient may be the residual protein moiety (1% or less) present in both the CWS and CMI fractions, as prepared in our laboratories.

The following observations support this conclusion:

a. CWS containing less than 1% protein were less active when combined with P3 and, most surprisingly, a combination of only P3 and PPD (75 µg each) produced a vigorous granulomatous inflammation in the lungs of mice and protected them against tuberculosis. Neither P3 nor PPD alone associated with oil droplets was active. Interestingly, this combination was completely ineffective in regressing established tumors unless CWS had been added (see NIAID-179). So far, the most potent P3-protein combination (3 log protection) was obtained with a protein sample extracted from BCG cell walls under mild conditions with aqueous butanol. This protection was 1 log higher than that afforded by 150 µg of original, untreated cell walls. Moreover, granuloma formation and protection were obtained even when P3 was combined with the dialyzable portion of the peptides (M.W. <10,000) split off from tuberculoprotein by acid hydrolysis.

b. The lipid-free but protein-containing, water-soluble mycobacterial adjuvant and antitumor fraction, MAAF (see NIAID-179), was not active in producing granuloma or protection, unless combined with P3.

c. Cell walls of M. kansasii, which contain P3 and share protein antigens with M. tuberculosis and M. bovis, protected mice against infection with H37Rv. Cell walls from M. smegmatis, which share some antigens but lack P3, were ineffective but conferred marked protection when combined with P3 (2 log reduction of H37Rv cells in the lungs of mice).

d. To date, protection induced in mice by intravenous administration of CW vaccines has always been dependent upon extensive granulomatous inflammation (as measured by lung weight increase). With vaccines consisting of original untreated cell walls, the protection conferred amounted to a 4 to 5 log reduction in H37Rv count, which was paralleled by a 2-fold increase in lung weight. A commercially available lyophilized viable BCG Tice T94 vaccine supplied by Drs. Rosenthal and Crispin which had been used for many years at RML as a standard provided a 2 log reduction in H37Rv count when administered IV at the optimal dose of 300 µg (moist weight) containing about 10^7 viable units, and this protection was accompanied by a low-grade granuloma formation. Recently, 2 new commercial viable vaccines, BCG Tice IL 105 and Montreal 1475-7, gave protection equal to that afforded by cell walls, but this was

accompanied by a lung weight increase similar to that caused by the cell walls. In agreement with recent observations by others, we found that the lyophilized BCG Tice T94 had a high content of nonviable "insoluble" and "soluble" antigenic material. This excess antigen may interfere with induction of cell-mediated immunity by causing antibody formation. In this respect, use of BCG Tice T94 vaccine could be advantageous inasmuch as the resulting humoral response may have a suppressive action on cell-mediated hypersensitivity and hence combat some deleterious effects of this chronic inflammatory reaction at the expense, of course, of a high level of protection.

Presumably, cell walls associated with oil droplets augment cell-mediated immunity like viable BCG vaccines that are essentially devoid of nonviable antigen. Conceivably, the state of hypersensitivity induced by CW-oil droplet vaccine could be modulated by a controlled humoral response induced with defined bacillary antigens and/or antibody enhancing adjuvants incorporated into the aqueous phase of the vaccine.

4. Components of mycobacteria involved in in vivo production of lymphokines. Collaboration with Drs. Salvin and Youngner in studies dealing with fractions of mycobacteria responsible for in vivo production of migration inhibition factor (MIF) and interferon, and their relationships to such diverse properties as protection from viral infection and tumor immunity has been continued. Last year we reported that these soluble mediators have been induced in high titer in the circulation of mice by intravenous injection of BCG CW vaccines, followed after an appropriate interval by IV challenge with old tuberculin. As in resistance to pulmonary tuberculosis, cell walls can be replaced by a combination of P3 and PPD for sensitization for production of MIF; addition of adjuvant-active CWS did not augment this response. Neither PPD nor P3 alone sensitized mice for MIF production. The demonstrated effectiveness of P3 to promote cellular immunity to tuberculoprotein is further supported by the finding that M. smegmatis cell walls, which do not contain P3, did not immunize for in vivo MIF release whereas walls from M. kansasii, which do contain P3, were active in this respect.

Significance to biomedical research and the program of the Institute:

In every instance in which our mycobacterial components immunized mice for in vivo MIF production, these same components effectively immunized mice against tuberculosis. This correlation suggests that circulating mediator(s) in MIF-active sera play an instrumental role in the cell-mediated immune process. From the chemically complex tubercle bacillus, we have isolated 2 classes of substances, one a protein or its peptide fragments, and the other mycolic acid esters. When these components were physically combined and associated with minute oil droplets, an antigen-adjuvant system that strongly stimulated cellular immunity resulted. We have been experimenting with PPD as a convenient source of the protein antigen.

Of these high molecular weight fatty acid esters, the most powerful in enhancing delayed hypersensitivity in mice to the protein antigen is P3, rather than CWS. Surprisingly, regression of established guinea pig tumors was achieved by inoculating them with cell walls attached to oil droplets, but not with a combination of P3 + PPD unless a small quantity of CWS (itself inactive) was added (see NIAID-179). The reason for this different behavior to different bacillary structures is a complex matter and is presently under investigation.

The bioassays used in this project will continue to be invaluable in the search for the components of mycobacteria responsible for either enhancement or inhibition of tumor growth. Such studies aid in the elucidation of the molecular basis of the immunopotentiating action of BCG or mycobacteria in general. By learning more about which antigens are important and how to present them to the host, it should be possible to obtain balanced cellular and humoral responses and thereby devise a useful nonliving antituberculosis vaccine.

Proposed course of project:

We are continuing our efforts to determine the exact structure of P3 in order that it may be possible to synthesize it. Compounds, like P3, consisting of mycolic acids esterified to sugars other than trehalose and possessing similar biological activities are being accumulated in quantities sufficient for detailed chemical and biological analysis.

Further studies are planned to determine if the MIF-active sera from mice immunized with the oil droplet-PPD-P3 complex possess other lymphokine properties (e.g., lymphotoxin, chemotactic factor, skin-reactive factor) and interferon.

Attempts are in progress to synthesize a peptide chain made up of those 5 amino acids presumed to be responsible for the activity attributed to the protein remnants in CWS. The sequence of these amino acids will be the same as that determined in a skin test-active tuberculoprotein. We intend to covalently bind or couple this synthetic peptide to P3 and thereby even more effectively augment cell-mediated immunity.

Honors and Awards:

Dr. D. Granger

Invited to participate in the Eighth Annual Tuberculosis Conference sponsored by the Tuberculosis Panel of the U.S.-Japan Cooperative Medical Science Program in New Orleans, Louisiana, October 1973.

Invited to present a paper at the Second Annual Collaborative Conference of the Carcinogenesis Program sponsored by the National Cancer Institute, San Antonio, Texas, November 1973.

Dr. E. Ribi

National ASM Lecturer.

Editorial Board Member, Infection and Immunity.

Reviewer for Journal of Infectious Diseases, Journal of the National Cancer Institute, American Review of Respiratory Diseases, and Journal of Chromatographic Science.

Invited (with Drs. Parker and Milner) to contribute a chapter on "Microparticulate gel chromatography accelerated by centrifugal force and pressure" in Methods of Biochemical Analysis. (See NIAID-179).

Invited to present a paper at the Symposium on "Chemical and cellular aspects of immunity to tuberculosis" at the 12th International Tuberculosis Conference, Tokyo, Japan, September 1973.

Invited to present a paper at the Eighth Annual Tuberculosis Conference sponsored by the Tuberculosis Panel of the U.S.-Japan Cooperative Medical Science Program in New Orleans, Louisiana, October 1973.

Invited to present a paper at the Second Annual Collaborative Conference of the Carcinogenesis Program sponsored by the National Cancer Institute, San Antonio, Texas, November 1973.

In press:

Ribi, E., Azuma, I., Meyer, T. J., Parker, R., Brehmer, W., and Yamamura, Y.: Chemical structure and biological functions of mycobacterial cell walls with special reference to immunity against airborne infection with M. tuberculosis strain H37Rv. Bull. Int. Union Tuberc.

Meyer, T. J., Anacker, R. L., and Ribi, E.: effects of pretreatment of mice with BCG cell walls in saline on subsequent vaccination with BCG oil droplet vaccine. Cellular Immunity.

Serial No. NIAID-178
1. Rocky Mountain Laboratory
2. Molecular Biology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Structure and biologic activity of endotoxins

Previous Serial Number: Same

Principal Investigator: K. C. Milner

Other Investigators: J. E. Cutler (NIH Postdoctoral Fellow)

Cooperating Units: Dr. Jacob Fine, Harvard Surgical Unit, Boston City Hospital, Boston, Mass.
Dr. J. A. Rudbach, University of Montana, Missoula
Dr. J. R. Vestal, University of Cincinnati, Ohio
Dr. Claes Weibull, University of Lund, Sweden

Man Years	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	3 10/12			5
Professional:	10/12		1 2/12	2
Other:	3			3

Project Description:

Objectives:

To investigate the features of composition and structure of bacterial endotoxins (ET) required for expression of characteristic host reactivity; to establish and standardize improved methods for the quantitative bioassay of endotoxins and similar materials; and to study the mechanisms of the biological effects involved.

Methods employed:

Diverse physical, chemical and immunological methods are used, with current emphasis on bioassay and statistical analysis.

Major findings:

1. For the purpose of defining the utility of the limulus amebocyte lysate test for ET, we have extensively compared it with other assays and have applied the tests to a variety of materials, including typical ET extracted from enteric gram-negative bacteria, extracts of polysaccharide-deficient mutants, anomalous lipopolysaccharides (LPS) from species of Brucella and Thiobacillus, synthetic polynucleotides, and extracts of Candida albicans. Most of the tests were repeated many times so comparisons could be made in terms of means and standard errors. Furthermore, in all limulus tests of the various materials (and wherever else possible), a standard preparation of typical ET was included to permit estimates of relative potency and thus to facilitate comparisons between tests.

We found, as before, that for ET from Enterobacteriaceae, potency in the limulus test correlated well with the results of standardized tests for pyrogenicity and lethality. Among the various substances tested, only bacterial LPS were highly potent in clotting limulus lysate. However, it was disappointing to learn that among the "anomalous" LPS there was little correlation between limulus potency and the results of other tests. In fact, one LPS from Thiobacillus ferrooxidans (adapted to cultivation on glucose as energy source), which was very weakly pyrogenic and lethal, was fully as potent in the limulus test as the standard LPS from Salmonella enteritidis. Also, contrary to expectation, bovine pancreatic ribonuclease (which is supposed to attack only single-stranded RNA) was found to hydrolyze synthetic double-stranded polynucleotides and produce some decrease in the limulus potency. Additionally, an extract of C. albicans, which was devoid of pyrogenicity for rabbits in doses up to 15 milligrams, was found to clot limulus lysate.

2. Dr. Cutler has continued to study fractions of C. albicans for ET-like effects on experimental animals and also for other physiological and immunological effects. Significant findings include:

a. Phenol-water extraction of C. albicans previously had yielded a substance, mainly polysaccharide, with weak endotoxin-like properties. Further refinement on a Sepharose column increased the lethality of the material to actinomycin D-treated mice but left it nonpyrogenic for rabbits, even in large doses (15 mg). This differentiates the material from bacterial ET, which would be active in both systems, and rules out contamination with ET as the source of activity.

b. Ultracentrifugation of the extract showed it to be a macromolecular complex (>10^6 daltons), which dissociated under the influence of sodium deoxycholate (NaD) and reassociated upon removal of the NaD. Nevertheless, the dissociated material would not hybridize with ET dissociated by NaD (method of Rudbach et al., 1967), another indication of a fundamental difference from bacterial ET.

c. A second toxin, both lethal and pyrogenic, had been isolated from C. albicans by a laborious procedure beginning with extraction with ethylenediamine. It was discovered that such a toxin could be isolated by direct treatment of whole cells or cell walls with Pronase. The further observation that treatment with Pronase of the toxin described above (a,b) rendered it pyrogenic as well as lethal, and made it appear that the enzyme was activating a latent toxin, either by unmasking reactive groups or by contributing something of its own substance to the fungal product. The Pronase itself had no endotoxin-like activity.

d. The observation that either active candidiasis or inoculation with toxins from C. albicans produced an acute inflammatory response with infiltration of polymorphonuclear leukocytes led to a screening of methods for measuring chemotaxis. Eventually, a new agar-plate method was devised, which would appear to have wide applicability. It is much simpler and less expensive than the Boyden-chamber method that has been used most extensively. A paper on the technique will be presented at a national meeting in May.

3. a. In a report (abstract) on a "Dialyzable Toxic Product of Serratia marcescens", J. M. Quarles et al. incorporated collaborative results obtained here. The tentative conclusion was that the small molecular weight toxic product not filtered out by the kidney machine was probably not ET, although traces of ET occasionally appeared as a contaminant.

b. Dr. Weibull has shown, by electronmicroscopy, clear structural differences between ET from wild-type cultures and polysaccharide-deficient mutants of S. typhimurium and S. minnesota. A manuscript has been prepared for publication.

c. Under contract with FDA, Dr. Rudbach has produced a large quantity of ET for national distribution as a reference standard, particularly for control of the limulus test. Individual batches have been chosen for pooling on the basis of tests conducted here, and the final pool is now being examined intensively.

Significance to biomedical research and the program of the Institute:

1. The possible clinical significance of the limulus test was mentioned in last year's report. Our recent results demonstrate that the test is unusually, if not quite absolutely, specific for bacterial LPS.

If rapid detection of ET in body fluids or tissues proves to be of great value, this should be the test of choice, for both sensitivity and specificity. It has also been hoped that the test would be pharmacologically useful for control of materials for injection—in particular that it could replace the expensive and time-consuming rabbit test for pyrogenicity. Our finding that some very weakly endotoxic LPS from nonenteric sources were maximally potent in the limulus assay militates against this use of the test. There would be too many false positives; i.e., the rejection rate would probably be much higher than with the rabbit test, and higher than would be warranted by the potential effects in human beings.

2. Several fungal toxins are strong poisons and/or carcinogens. These, however, derive mostly from saprophytic forms. The role of toxins in superficial or systemic mycoses is essentially unknown. Although the toxic components of C. albicans are weak, they could have pathogenic importance because of the great proliferation of the fungus in certain types of infection. Moreover, these and other components elicit cellular and immune responses other than those attributable to toxicity. An improved technique devised for studying one such response (chemotaxis) should have broad applicability.

Proposed course of project:

1. Several aspects of the reaction between bacterial LPS and lysed amebocytes of Limulus polyphemus are believed to be worthy of continued investigation and suited to our capabilities. Further evaluation of the specificity of the reaction is essential to clinical interpretations, and we are actively pursuing this study in collaboration with other units. Drs. E. T. Yin, S. Watson, and J. Fine have all found means for increasing the sensitivity of limulus lysate through removal of inhibitors. We have been asked to evaluate such methods independently. The complete chemical analysis and bioassay of a reference ET for the limulus test will also begin shortly. Related to the foregoing is a need to study the reaction between limulus lysate and polynucleotides more completely and to determine the precise effects of ribonucleases on this reaction.

2. Continuing studies from previous years include: a) an examination of the effects of pretreatment with polysaccharide-deficient mutants of salmonellae on subsequent challenge with virulent culture; b) efforts to supply extracts from additional classes of mutant cultures to Dr. Weibull for study by electronmicroscopy; and c) continued collaboration with Dr. Vestal in studies of LPS from chemolithotrophic bacteria.

3. The apparent activation of Candida toxin by Pronase is to be studied in more detail. Further purification and characterization of the toxin will be attempted, and chemotaxis by fractions and whole cells of C. albicans will be particularly studied, as well as other physiologic and immunologic effects.

Honors and Awards:

Dr. K. C. Milner

Invited (with Drs. Ribi and Parker) to contribute chapter on "Microparticulate gel chromatography accelerated by centrifugal force and pressure" in Methods of Biochemical Analysis (in press, see project NIAID-179).

Invited to contribute a chapter on "Comparison with other tests" to a monograph on The Limulus Lysate Test for Detection of Endotoxin. Dieter Gröschel (ed.), Marcel Dekker, publisher.

Editorial Board Member, Infection and Immunity.

Reviewer for Journal of Infectious Diseases.

Faculty Affiliate (Lecturer in Medical Microbiology), University of Montana.

Dr. J. E. Cutler

Guest seminars at Duke University School of Medicine (October 1973) and University of Colorado Medical Center (January 1974).

Guest lecturer at University of Montana (May 1973 and October 1973).

Publications:

von Eschen, K. B., Rudbach, J. A., and Milner, K. C.: Endotoxin-altering activity in serum of developing chicken embryo: lack of correlation with embryonic susceptibility to injected endotoxin. Infect. Immun. 7: 683-684, 1973.

Milner, K. C.: Patterns of tolerance to endotoxin. J. Infect. Dis. Suppl. 128: S237-S245, 1973.

Vestal, J. R., Lundgren, D. G., and Milner, K. C.: Toxic and immunological differences among lipopolysaccharides from Thiobacillus ferrooxidans grown autotrophically and heterotrophically. Can. J. Microbiol. 19: 1335-1339, 1973.

Serial No. NIAID-179
1. Rocky Mountain Laboratory
2. Molecular Biology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Mycobacterial components in cancer immunotherapy

Previous Serial Number: Same

Principal Investigators: E. Ribi and D. L. Granger

Other Investigators: K. C. Milner and R. F. Smith

Cooperating Units: Dr. I. Azuma, Osaka University, Osaka, Japan
Dr. W. Brehmer, Robert Koch Institute, Berlin, Germany
Dr. J. Gutterman, Anderson Hospital and Tumor Inst., Univ. of Texas, Houston
Dr. T. J. Meyer, Univ. of Colorado Med. Center, Denver
Dr. R. Parker, Hamilton Biochemical Research Lab., Hamilton
Mr. S. M. Strain, Montana Lung Assoc. Fellow, Hamilton
Drs. B. Zbar and H. Rapp, NCI, NIH

Man Years	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	6 11/12			8 3/12
Professional:	1 10/12		1 4/12	3 2/12
Other:	5 1/12			5 1/12

Contract Support:

Contract Number	Project Officer (NIAID, NCI)	Contractor	Contractor's Project Director	Annual Funding Level
Still under negotiation	H. Rapp E. Ribi	Hamilton Biochemical Research Lab.	R. Parker	about $60,000

Project Description:

Objectives:

Primary goals are elucidation of the fine structure and chemical composition of microorganisms and correlation of these attributes with certain biologic properties of cells or cell fractions, and the development of new tools and techniques and their application to current problems.

Studies this year again have been concerned with 1) isolation and structural identification of mycobacterial cell wall preparations responsible for the enhancement of specific tumor immunity (suppression of growth and regression of established tumors), and 2) applying micro-particulate gel chromatography accelerated by centrifugal force or pressure for separating complex mixtures of mycobacterial lipids.

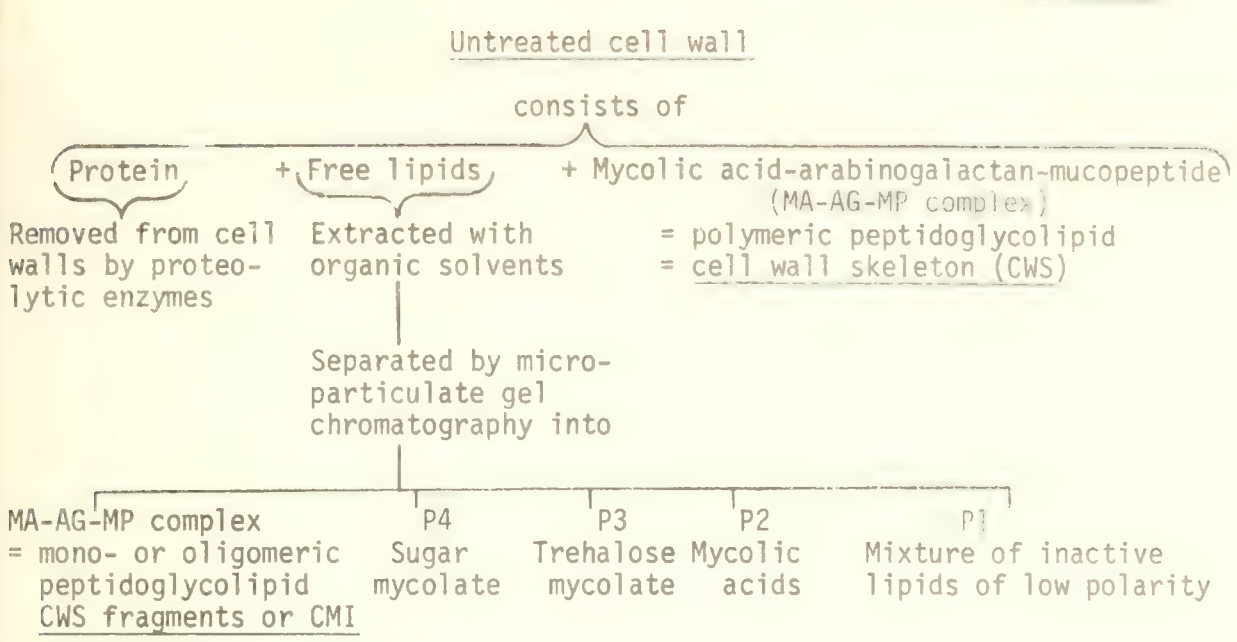
Methods employed:

A combination of biophysical, chemical, and immunological approaches was used.

Major findings:

1. We reported last year that intradermal growth of a transplantable hepatocarcinoma in syngeneic guinea pigs was suppressed when 10^6 tumor cells, together with 40-μg quantities of BCG cell walls attached to minute oil droplets, were inoculated. We also reported that injection of 300-μg of BCG cell walls attached to oil droplets into established skin tumors of animals inoculated 7 days before with 10^6 tumor cells caused about 60% of tumors to regress and prevented growth of metastases. Animals in which tumor growth was suppressed or in which established tumor regressed acquired systemic tumor immunity. Comparable effective doses of BCG were 6×10^6 and 24×10^6 viable units for tumor suppression and regression, respectively.

2. Attempts were continued to determine which cell-wall components described in the scheme below can be used in place of viable BCG in *in vivo* tumor immunology.


Untreated cell wall
consists of
Protein
+ Free lipids
+ Mycolic acid-arabinogalactan-mucopeptide (MA-AG-MP complex)
Removed from cell walls by proteolytic enzymes
Extracted with organic solvents
= polymeric peptidoglycolipid
= cell wall skeleton (CWS)
Separated by microparticulate gel chromatography into
MA-AG-MP complex = mono- or oligomeric peptidoglycolipid CWS fragments or CMI
P4 Sugar mycolate
P3 Trehalose mycolate
P2 Mycolic acids
P1 Mixture of inactive lipids of low polarity

P3 consists of fatty acid esters in which the sugar, trehalose, is esterified with at least 3 types of mycolic acid, 2 of which were previously unknown. The same 3 types of mycolic acid are present in the cell wall skeleton (CWS) and in its "fragments" (CMI).

With more syngeneic guinea pigs available through the courtesy of Dr. B. Zbar, NCI, it was possible to repeat previous experiments with animals in sufficient number to permit statistical analyses. These provided reliable data demonstrating that P3 in addition to the cell wall skeleton was necessary for regression of established tumors. This parallels mouse antituberculosis immunity and granuloma formation data where maximal effect is obtained only with this combination of components. However, in tumor suppression CWS and CMI are effective but P3 has no additive effect. These materials also produce EAE in guinea pigs.

3. CWS prepared according to the above scheme still contained some tuberculoprotein, the amount of which varied from batch to batch. It appears that about 1% protein content is essential for CWS to cause optimal tumor suppression and for CWS + P3 to cause optimal regression. When CWS contained only about 0.5% tuberculoprotein, the deficiency could be corrected by adding PPD. Neither P3 nor PPD alone was effective in causing tumor suppression or regression.

Accumulating evidence indicates that the tuberculoprotein remaining in CWS may represent the specific antigen which produces delayed hypersensitivity (DH) or cell-mediated immunity. P3 possesses a stronger adjuvant action than the mycolic acids that are bound to the polysaccharide-mucopeptide complex in CWS or its fragments. In

combination with a protein(s) of the tubercle bacillus it evokes a vigorous DH reaction of which granulomatous inflammation may be the most dramatic end result. In established tumors, DH may kill many tumor cells as "innocent bystanders". Since there are fewer tumor cells to contend with in suppression than in regression, induction of immunity by CWS alone may be sufficient in one system but not in the other.

4. Cell walls from virulent Mycobacterium tuberculosis, virulent M. bovis, and M. kansasii were at least as effective in regressing established dermal tumors and in preventing growth of lymph node metastases as were cell walls from attenuated M. bovis strain BCG. Cell walls from all of these organisms contained P3. Those from avirulent M. smegmatis which do not contain P3 were not effective but were rendered effective in tumor regression when combined with P3 isolated from cells of M. tuberculosis. Since the structure of the cell wall of M. smegmatis resembles that of BCG, we expected to find that the former was also active in tumor suppression.

5. The lipid-free (no mycolic acid), water-soluble mycobacterial adjuvant and antitumor factor (MAAF) described by Hiu was also capable of regressing tumors, but only after being mixed with P3. MAAF alone enhances tumor growth. It also contains protein to which DH or cell-mediated immunity is enhanced by the adjuvant action of P3. Thus, when MAAF and P3 were combined and associated with oil droplets, DH to PPD was produced in guinea pigs, even though neither of these products alone induced PPD sensitivity.

6. We recently demonstrated that tumor growth was enhanced when certain mycobacterial components, such as MAAF or acetylated cell walls, were tested in the suppression test. Measurement of tumor size during these experiments revealed highly significant differences between control and experimental groups. Preparations producing this phenomenon share the immunological properties of being effective adjuvants for humoral antibody production against unrelated antigens (e.g., bovine serum albumin, sheep red blood cells) and yet by themselves are incapable of eliciting a Koch phenomenon in the skin of guinea pigs. It is possible that in addition to being inactive in the suppression test (i.e. no induction of a primary cell-mediated immune response and hence no killing of tumor cells as "innocent bystanders"), these adjuvants promote a humoral response to the tumor-specific transplantation antigen which interferes with the feeble tumor graft rejection of this poorly antigenic carcinoma (tumor line-10). This complex interaction between host, adjuvant, and neoplastic cell might, therefore, result in a more rapidly developing tumor.

Reversal of the tumor enhancement effect with resulting tumor suppression could be accomplished in the case of MAAF by adding P3, and in the case of the acetylated BCG cell walls by adding tuberculoprotein (PPD).

Acetylated cell walls show adjuvanticity when combined with various antigens but alone are incapable of producing cell-mediated immunity in the mouse lung.

Significance to biomedical research and the program of the Institute:

Nearly all published data suggest that the most effective method of destroying tumors is to stimulate a cell-mediated cytodestructive mechanism and concurrently suppress any associated humoral response that would interfere with cell-mediated immunity. Several investigators have found that viable BCG prevents tumor formation, delays rate of tumor growth, and regresses established tumors. It has also been found to enhance tumor growth. Most substrains of BCG used in the preparation of antituberculosis vaccines differ in morphologic, biochemical, immunogenic, and allergenic properties, apparently because conditions under which they are cultured vary. It is not surprising, therefore, that their antitumor activity also varies. For example, it has been demonstrated that modification of cultural conditions of a given BCG substrain led to selection of 2 different phenotypes, one of which enhanced tumor growth while the other inhibited it. The parental substrain had no detectable influence on tumor growth, presumably because of the counteracting effect of the 2 phenotypes. A similar process of selection could conceivably occur in vivo, i.e., when any substrain of BCG is injected into a tumor it could result in either inhibition or enhancement of its growth or have no effect at all. Preliminary findings reported here may provide some explanation for these opposite effects and suggest that the cellular and humoral responses might be manipulated with vaccines containing varying quantities of defined components of BCG. Since we found that the degree of tumor regressive potency of a vaccine is paralleled by the amount of P3 present, and P3 is capable of counteracting enhancement of tumor growth by other bacillary constituents, it is anticipated that the structural ingredients can be presented to the host in a manner calculated to augment cell-mediated immunity.

Proposed course of project:

The need for careful and extensive studies on cancer immunotherapeutic agents becomes poignantly evident when we consider that strategies intended to benefit a cancer patient may in fact result in an acceleration of the disease process. Therefore, we plan to 1) chemically and structurally characterize recently discovered tumor-enhancing mycobacterial components, 2) define their mode of action, 3) find ways in which P3 can be employed to most efficiently counteract the enhancing effect, and 4) study the action of P3 in facilitating tumor graft rejection.

The enzyme studies proposed in last year's report had to be discontinued due to the press of other work. Dr. Parker demonstrated

marked differences in isozyme patterns in liver, muscle, and serum of normal and line-10 tumor-bearing guinea pigs and it is hoped that this work can be pursued again during the coming year.

Publications:

Ribi, E., Smith, R. F., Strain, S. M., Petcoff, D. G., Parker, R., and Goode, G.: Purification of Δ^9-tetrahydrocannabinol, an active constituent of marihuana, by accelerated microparticulate gel chromatography. Prep. Biochem. 3: 209-220, 1973.

Abinanti, F. R.: Comments on immune response to antigens of inactivated microbial agents. J. Am. Vet. Med. Assoc. 163: 815-816, 1973.

Azuma, I., Ribi, E. E., Meyer, T. J., and Zbar, B.: Biologically active components from mycobacterial cell walls. I. Isolation and composition of cell wall skeleton and component P_3. J. Natl. Cancer Inst. 52: 95-101, 1974.

Meyer, T. J., Ribi, E. E., Azuma, I., and Zbar, B.: Biologically active components from mycobacterial cell walls. II. Suppression and regression of strain-2 guinea pig hepatoma. J. Natl. Cancer Inst. 52: 103-111, 1974.

Zbar, B., Ribi, E., and Rapp, H. J.: An experimental model for immunotherapy of cancer. Natl. Cancer Inst. Monogr. 39: 3-7, 1973.

Ribi, E. E., Meyer, T. J., Azuma, I., and Zbar, B.: Mycobacterial cell wall components in tumor suppression and regression. Natl. Cancer Inst. Monogr. 39: 115-119, 1973.

In press:

Zbar, B., Ribi, E., Meyer, T. J., Azuma, I., and Rapp, H. J.: Immunotherapy of cancer: regression of established intradermal tumors after intralesional injection of mycobacterial cell walls attached to oil droplets. J. Natl. Cancer Inst.

Ribi, E., Parker, R., and Milner, K. C.: Microparticulate gel chromatography accelerated by centrifugal force and pressure. In Methods in Biochemical Analysis, Vol. 22.

Serial No. NIAID-180
1. Rocky Mountain Laboratory
2. Epidemiology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The encephalitides

Previous Serial Number: Same

Principal Investigator: L. A. Thomas

Other Investigators: J. E. Coe

Cooperating Units: Dr. Hugh Keegan, Dept. of Preventive Medicine, Univ. of Mississippi School of Medicine, Jackson
Dr. David B. Lackman, Microbiology Laboratory, Montana State Dept. of Health, Helena

Man Years	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	1			1
Professional:	3/12			3/12
Other:	9/12			9/12

Project Description:

Objectives:

1) To investigate the role of garter snakes in the epidemiology of Western equine encephalitis (WEE), 2) to determine if blood-sucking leeches are capable of becoming infected with selected arboviruses and transmitting these viruses by refeeding, 3) to establish identification of field isolates from both mosquitoes and ticks, and 4) to detect arbovirus activity in human and animal populations and mosquitoes as measured by virus isolation and/or surveillance for antibodies.

Methods employed:

Standard virological laboratory procedures were used throughout the investigation which involved a variety of methods of testing.

Major findings:

1. Antibody response in garter snakes. Studies to detect antibody response in garter snakes by employing an indirect neutralization test are in progress. Specific antibody was measured in tests of pre- and post-inoculation sera from snakes collected from the Bear River Wildlife Refuge Area, Brigham City, Utah; however, there is a wide variation in antibody development among individual snakes exposed to the same dose of WEE virus.

2. Significance of leeches in transmissing virus. Four water buffalo leeches (Hirudinaria manillensis) fed to completion on an 8-week-old swine infected with Japanese B encephalitis virus (blood virus titer $10^{-3.7}$ suckling mouse LD_{50}) on 11-10-72 and on WEE-infected rabbits (blood virus titer range from $10^{-1.5}$ to $10^{-4.1}$) on 2-22-73. These leeches fed 4-2-73 on a normal 8-week-old swine. We were unable to detect any evidence of viremia or antibody response in the swine. The largest of these 4 leeches was dissected and various parts of the body were tested for virus, but none was detected. These results indicate that the water buffalo leech is not involved in the epidemiology of WEE or Japanese B viruses.

3. WEE and St. Louis encephalitis (SLE) virus activity in Montana. Surveillance for WEE and SLE activity in Montana and neighboring states is maintained through a cooperative program with local physicians and the Montana State Department of Health. In a survey initiated in 1973, 4,268 sera, representing 13 counties, were tested. Of these, 179 (4.19%) were positive for WEE and 169 (3.96%) were positive for SLE. Among individual counties the rates for WEE varied from 3.4% to 13% and that for SLE varied from 2% to 13%. Of 338 sera received from North Dakota, 36 were HAI positive for WEE and 11 were positive for SLE.

Observations made during the summer of 1973 suggest little activity of WEE or SLE viruses. About 200 mosquitoes from Fort Shaw, Montana were tested and found free of virus. However, 5 sentinel chickens in the area became seropositive to WEE. The small number of sera submitted for diagnostic serology suggests that few cases of encephalitis occurred among man or horses.

Significance to biomedical research and the program of the Institute:

The employment of the indirect neutralization test in studies to determine the capability of garter snakes to develop antibodies following

experimental WEE virus infection is under investigation and has definite promise of being a suitable method for measuring neutralizing antibodies in these cold-blooded vertebrates.

Proposed course of project:

The study of WEE antibody response in experimentally infected garter snakes will continue. When convinced of the reliability of the test, field collected sera will be tested to determine what role, if any, garter snakes play in the epidemiology of WEE virus. The laboratory will continue to be of service to the Montana State Department of Health and other agencies in the isolation of virus and serological testing for antibodies (both mosquito- and tick-transmitted viruses). Efforts will be continued to obtain, produce, and test reagents for serological testing as well as employ the best methods in testing.

Serial No. NIAID-181
1. Rocky Mountain Laboratory
2. Medical Entomology and Acarology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Tick-borne disease agents

Previous Serial Number: Same

Principal Investigators: C. M. Clifford, C. E. Yunker, and L. A. Thomas

Other Investigators: J. E. Keirans and R. N. Philip

Cooperating Units: Dr. J. Casals, Yale Arbovirus Research Unit, New Haven
Dr. E. Easton, University of Dar Es Salaam, Tanzania
Dr. J. E. George, Texas Technological College, Lubbock
Mr. R. Gresbrink, Oregon State Board of Health, Portland
Mr. Robin Rice, Dept. of Zoology, Univ. of Texas, Austin
Mrs. Margaret Sweeney, Idaho State Dept. of Health, Boise
Dr. Gerald Walton, University of Cork, Ireland

Man Years	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	3 9/12			3 9/12
Professional:	1 1/12			1 1/12
Other:	2 8/12			2 8/12

Project Description:

Objectives:

To determine the incidence, distribution, and ecology of tick-borne diseases that influence man and domestic animals in the United States. Of equal importance is the investigation of the vector relationships of the increasing number of viruses known or thought to be associated with ticks.

Methods employed:

Field and laboratory approaches include conventional and specialized entomological, ecological, virological, and immunological techniques.

Major findings:

1. Viruses in ixodid ticks. "86" virus, an undescribed agent from Ixodes uriae in Oregon, was characterized with regard to certain biochemical, biophysical, serological, and pathological attributes. It is capable of producing a moderate cytopathogenic effect (CPE) and plaques in Vero cells, is markedly affected by treatment with ether and sodium desoxycholate, stable in the presence of 5-bromodeoxyuridine, unaffected by pH3, and of a size ranging between 100 to 200 mμ. In complement fixation (CF) test its sole relationship was to Sakhalin virus (from I. uriae in eastern Russia), but serum neutralization (N) test showed it to be specifically distinct. These reactions tend to place "86" virus in a unique position as an enveloped RNA virus resistant to acid pH. Further characterization is planned. A related strain from Alaska (71.1628) is also under study.

Sera from seabirds from Yaquina Head, Oregon, were tested by hemagglutination inhibition (HI) test for evidence of infection with Tuleniy virus. Nineteen of 24 murres, including some newly hatched chicks, were serologically positive, and 5 gull chicks were negative. The presence of antibodies to Tuleniy virus in newly hatched chicks implies transfer of maternal antibody, perhaps in the eggs. The high percentage of murres reacting in the N test poses the question of how Tuleniy virus is circulated among these birds which are predominantly immune during the 3-month nesting season and at sea the rest of the year. In tests of tissues of these birds for viruses, Tuleniy virus was not recovered, but 2 strains of Yaquina Head virus were recovered in suckling mice and Vero cells.

The close serological relationship of Tuleniy virus and Japanese B encephalitis (JBE) has prompted speculation about the evolution of the former from a JBE-like ancestor to a variant capable of overwintering in ticks and emerging in epidemic form in mosquitoes. Because of this speculation and because JBE virus can be transmitted by at least 8 North American mosquito species, it seemed advisable to determine if Tuleniy virus (Three-Arch strain) could be transmitted by an efficient vector of JBE virus. In 4 experiments, Culex tritaeniorhynchus, the principal vector of JBE virus, was intrathoracically inoculated with Tuleniy virus in various concentrations and ground and inoculated singly into suckling mice at intervals. Average virus content of mosquitoes rapidly diminished until, by day 7, virus could no longer be detected. Additional tests are in progress, but these results suggest that this efficient vector of JBE virus is refractory to Tuleniy virus. If true, we must conclude that JBE and Tuleniy viruses are, in spite of antigenic similarities, biologically distinct entities whose evolutionary relationships are unclear.

Viremia experiments were conducted with Tuleniy and Yaquina Head viruses in wild-caught seabirds to evaluate their possible role in the natural cycles of the viruses. One of 10 baby murres and 2 of 2 adult murres had low levels of Tuleniy virus 4 days after subcutaneous inoculation. In view of the large number of wild-caught murres later found serologically positive to this virus, such a low percentage of susceptibles might be expected. Seagull chicks were able to circulate virus for 6 days. However, titers were low and, although all 5 birds tested were viremic at one time or another, viremia was not prolonged. Similarly, Yaquina Head virus caused a brief viremia of low titer in seagull chicks.

Suckling mice, as well as newly hatched chicks and domestic ducks inoculated by 2 different routes, were shown to be poor donors of circulating Tuleniy virus. Therefore, until a satisfactory host can be found, this virus can be offered to candidate vectors only by artificial means (infected meal or intrathoracic inoculation).

Sera collected in 1960 from Alaskan natives by Dr. R. Philip were tested for Kemerovo and Sakhalin group viral antibodies. In mouse N tests, no reactors to "86" virus were detected. In plaque N tests of about 500 sera from Alaskan males, 4% showed significant protection against the Yaquina Head-like agent RML 57493-71.

Isolation in Vero cells of CTF virus from human bloods was continued. This study is being done in collaboration with the Idaho State Department of Health. (See also NIAID-182 and NIAID-188).

2. Viruses in argasid ticks. A unique strain of virus isolated in 1969 from Argas cooleyi in Texas was shown to share many of the biochemical and biophysical characteristics of "86" virus from I. uriae, but thus far has failed to cross react in N or cross CF tests with "86" or any of 120 other arboviruses. The virus and its serological reagents have been sent to Dr. Jordi Casals (YARU) for comparison with viruses not accessible to us. If no relationships can be shown with any of these, it will be described as new.

Four isolations of virus were made in Vero cells from 17 pools of Ornithodoros sp. (capensis group) from Hawaii. Three of these isolates were mixed containing 2 or 3 agents of the serological groups, Hughes, Quaranfil, and Nyamanini. The pure isolate (RML 63668-9) is a member of the Nyamanini group, probably Midway or Green Kure virus. Both of these viruses are known from this tick in the Central Pacific.

Midway and Green Kure viruses were shown to differ in development and morphology of their plaques in Vero cells, as well as in size of their infectious particles. However, attempts to separate these two in serological tests have thus far failed. Anti-Midway serum protects Vero

cells from infection with both agents, whereas that prepared against Green Kure virus is impotent against either virus. Further tests are in progress.

3. Assay systems for tick-borne viruses. Four tick-borne viruses, Manawa, Chobar Gorge, Zirqa, and Dera Ghazi Khan, were received from YARU and added to our bank of viral agents. These four were tested in tissue cultures and the last three were found capable of producing CPE and plaques in Vero cells.

Relationships among agents of certain serological groups of tick-borne viruses remain obscure because of low infectivity in mice and weak reactivity of antigens and antisera in CF and N tests. Much effort was expended on developing reliable CF and N test procedures by which to identify and classify these agents.

Results of CF tests with Hughes group agents showed that Sapphire II and Soldado viruses can readily be distinguished from each other and from other members of the group. Farallon and Zirqa viruses are likewise readily separable, although some cross reactivity is evident. Hughes, Raza, and Punta Salinas viruses were so closely related they were indistinguishable in this test. N test (plaque-reduction) results confirm these patterns but demonstrate a broader cross reactivity for certain members than is evident by CF test.

Eleven members of the Kemerovo group were also studied by cross CF and plaque-reduction N tests. Here, N test was much more specific than CF in distinguishing among different virus strains. This specificity and the avidity of neutralization could be increased by incubation of virus-serum mixture at 4°C for 24 hours before inoculation. Other viruses of various serological groups, including CTF virus, as well as a member of unidentified agents originating from field-collected materials, were also more easily studied in N tests when thus treated.

Significance to biomedical research and the program of the Institute:

The genus Ixodes plays an important role in the transmission of viruses that cause illness in man, e.g., RSSE, Central European, Louping Ill, Kemerovo, Omsk haemorrhagic fever, and Powassan virus. They are also important vectors of pathogenic viruses to domestic livestock. Our demonstration of a multiplicity of viruses in Ixodes ticks in North America is of potential epidemiological significance.

Russian scientists have suggested that ixodid and argasid ticks play an important role in overwintering a number of arboviruses. The ability of mosquito-borne arboviruses to adapt to acarids would provide a foci of mosquito-transmitted viruses along the edge of their distribution. The presence of West Nile virus in ticks in areas where mosquitoes are absent is cited as support for this theory by these workers. They are also

interested in Tuleniy virus (isolated from I. uriae) because of its affinity with JBE virus. Further, they claim to have propagated this tick-borne virus in at least one species of mosquito. All our experiments with our Oregon strain of Tuleniy virus have failed to substantiate their hypothesis. More experiments are planned.

Serological evidence provided by tests performed at the RML and by Russian workers have demonstrated that some of the recently isolated tick-borne viruses may infect man.

Basic work on techniques that will allow us to identify and characterize some of the tick-borne viruses that are of low pathogenicity for suckling mice is still continuing. These studies, if successful, could make an excellent contribution to the field of virology.

We have continued to add to our bank of tick-borne pathogens and are now capable of identifying these viruses from almost all areas of the world.

Proposed course of project:

Isolation and characterization of viruses associated with ticks will continue. Emphasis will be placed on devising techniques to allow for antigenic comparison of viruses now on hand. We will continue to add to our reference stock of tick-borne and related viruses and to prepare serological reagents for these agents. Efforts will be continued to develop tissue culture techniques to facilitate identification of tick virus strains as they are recovered.

We will continue to determine the vector potential of mosquitoes for viruses associated with American ticks. However, as mentioned last year, all our tick-borne studies especially those requiring reared arthropods are awaiting the replacement of an attendant in the RML insectary. Other aspects of this program have been delayed by changes in the RML mouse colony.

Publications:

Thomas, L. A., Clifford, C. M., Yunker, C. E., Keirans, J. E., Patzer, E. R., Monk, G. E., and Easton, E. R.: Tick-borne viruses in western North America. I. Viruses isolated from Ixodes uriae in coastal Oregon in 1970. J. Med. Entomol. 10: 165-168, 1973.

Yunker, C. E., Clifford, C. M., Keirans, J. E., Thomas, L. A., and Cory, J.: Tickborne viruses in western North America. II. Yaquina Head, a new arbovirus of the Kemerovo group isolated from Ixodes uriae. J. Med. Entomol. 10: 264-269, 1973.

Serial No. NIAID-182
1. Rocky Mountain Laboratory
2. Epidemiology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The epidemiology of human infections of special interest

Previous Serial Number: Same

Principal Investigator: R. N. Philip

Other Investigators: E. A. Casper, L. A. Thomas, C. E. Yunker, R. A. Ormsbee, and W. Burgdorfer

Cooperating Units: Dr. J. J. Plorde, Veterans Administration Hospital, Seattle, Washington
Dr. D. Sexton, EIS Officer, Mississippi State Health Department, Jackson
Mrs. Margaret J. Sweeney, Idaho State Department of Health, Boise

Man Years	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	2 8/12			2 8/12
Professional:	1 2/12			1 2/12
Other:	1 6/12			1 6/12

Project Description:

Objectives:

The primary objective is to acquire basic information about the epidemiology and prophylaxis of human infections with pathogens of special interest to RML.

Methods employed:

Field investigations utilizing laboratory methods for isolating disease agents and detecting antibody responses to infection are

emphasized. The methods are standard epidemiologic, statistical, and diagnostic procedures. As the situation warrants, specialized laboratory techniques are used for diagnosis.

Major findings:

1. Colorado tick fever (CTF). (See NIAID-181). Studies have been continued on the nature of the prolonged cell-associated viremia in this disease. In 1973, 115 blood samples from 72 patients from Montana, Idaho, and Wyoming with illnesses resembling CTF were examined for CTF virus in suckling mice and Vero cell cultures. Sixty-one specimens from 36 persons were positive, of which 11 resided in the Bitterroot Valley. The assay systems were equally sensitive for primary isolation of virus.

Seventy virus-positive cases have now been examined for viremia at various times after illness onset. Virus was isolated from 99 of 100 clots obtained during the first 12 weeks, from only one of 13 specimens obtained between 13 and 20 weeks and from none of 12 specimens obtained after 20 weeks. Cell-associated antigenemia, demonstrated by direct immunofluorescence, persisted somewhat longer and has been detected for as long as 135 days.

Serum from clotted blood was also tested in Vero cells. Hemolysis of varying degree was present in many specimens submitted by mail. The longest time interval from illness onset to isolation of virus from serum was 51 days, but most sera were positive only if they were obtained within 2 weeks of illness onset.

Preliminary attempts have been made to identify the cell types in bone marrow and spleen involved during early experimental CTF infection of mice. Although fluorescing cells can be relocated by light microscopy, staining qualities are altered and specific identification has not yet been accomplished.

2. Rickettsial infections. (See NIAID-171). Usefulness of the micro-immunofluorescence (micro-IF) test in serodiagnosis of typhus and spotted fever infections was investigated. The procedure was that used for study of chlamydial infections. Slide antigens, obtained from Dr. Ormsbee, consisted of inactivated purified rickettsiae adjusted to a concentration of 100 μg of cells diluted in 3% normal yolk sac. Conjugates consisted of fluorescein-labelled antiglobulin or labelled antiserum to globulins of specific immunoglobulin class. Five groups of sera were tested. They included 1) sera from suspected CTF cases, 2) sera from RML employees who had spotted fever or typhus, 3) sera obtained in 1964 from Ecuador typhus patients, 4) sera obtained by Dr. Plorde from typhus patients in Ethiopia, and 5) sera obtained by Dr. Sexton from patients in Mississippi who had tick-associated febrile exanthemata.

The micro-IF test is a promising serodiagnostic procedure for rickettsial infections. Micro-IF, MA and CF tests correlated very well with one another in the serodiagnosis of typhus infections. The micro-IF test is quite sensitive and titers as high as 1:65,000 were observed.

In spotted fever the results are not as clear-cut. The micro-IF and CF results correlated well with each other but, for reasons unknown, not with findings in the MA test. In the Mississippi series, 46 patients had illnesses clinically compatible with spotted fever. Seventeen had diagnostic (≥ 4-fold) increases or high titers (1:64 to 1:65,000) of micro-IF antibodies in single sera, which indicated recent Rickettsia rickettsii infection. One had a low titer (1:32) of questionable significance.

The seronegative group was particularly interesting. Most of these individuals had tick-associated illnesses with characteristic spotted fever rash, yet convalescent blood samples obtained at optimum times were nonreactive in all serologic tests. Most of these persons were treated with rickettsiae-specific antibiotics, but illnesses were often severe and it is difficult to reconcile lack of antibody response to specific treatment. The possibility that a nonspotted fever agent was involved is being considered (see NIAID-171). Dr. Thomas tested the Mississippi sera for CF antibodies to 3 arboviruses (Saw Grass, Lone Star and Silverwater), none of which is known to be pathogenic for man but all of which have been recovered in this country from Dermacentor variabilis, Amblyomma americanum or Haemaphysalis leporispalustris ticks. None of the sera reacted to these agents.

IgM antibodies were detected in sera from most Ecuador and Ethiopian typhus patients and seropositive Mississippi spotted fever cases. Few RML staff members with laboratory-acquired infection had IgM responses. Most had received typhus and spotted fever vaccine at various times before illness. This finding is compatible with the generally held belief that IgM antibodies are transient and not recalled to secondary antigenic stimuli. Of interest also is the observation that neither heat nor disulfide-bond reducing agents influenced IgM titers in micro-IF reactive sera. This is contrary to what has been observed by others in the CF and MA tests. Presumably, only one combining site on each antibody molecule is necessary in order to demonstrate immunofluorescence.

Significance to biomedical research and the program of the Institute:

It is unlikely that true "chronic" viremia occurs in CTF. Current evidence indicates that infection of new red blood cells occurs only during the first 3 weeks after entry of the virus in the body. This time interval coincides with appearance of circulating antibodies. Virus is stabilized within red cells where it remains viable for slightly longer than 3 months. On the other hand, attrition of infected cells is similar

to that of normal cells, and presence of CTF antigen can be identified for a short time after viability of virus is lost. In this sense the viremia in CTF is a prolonged, but self-limited viremia.

This unusual manifestation of infection appears to be a characteristic of several other arboviruses, viz. bluetongue of sheep and epizootic hemorrhagic disease of deer. It is interesting that these viruses are members of the orbivirus group, the taxon to which CTF virus has been assigned and which includes also the Kemerovo group of tick-borne viruses. None of these agents, except CTF, is definitely known to be pathogenic for man. All are double-stranded RNA viruses relatively resistant to lipid solvents, and all are morphologically and morphogenetically similar. Perhaps tropism for developing red cells is also a characteristic common to this group.

Immunofluorescence is not new to rickettsial research. However, the micro-IF test has several advantages over previously used methods. Small quantities of reagents are used. The antigens are placed on microscope slides by pen-point. One slide can be used to test 8 dilutions of serum against 9 or more antigens. The reactivity against various antigens can be directly compared because the same drop of diluted serum covers all antigens. Endpoints, although subjectively determined, are reproducible with experience. The test is somewhat more laborious and time consuming than the CF procedure and probably will have wider application as a research tool than as a routine serodiagnostic procedure. It should be particularly useful in detecting antigenic differences in rickettsiae using typing sera of mouse or guinea pig origin.

Proposed course of project:

We will continue to investigate infection of blood-forming elements by CTF and related viruses. Of particular interest are the long-range effects, if any. We hope to obtain follow-up information particularly relating to hematologic disorders from persons who have had CTF locally in years past.

Use of the micro-IF test in the study of rickettsial infections will be continued. Unusually complete and precise clinical information was obtained by Dr. Plorde on the Ethiopian typhus cases. This will be particularly useful in evaluating serologic procedures. Hopefully, additional information can be obtained which will clarify the nature of the seronegative spotted fever-like illnesses occurring in Mississippi.

Honors and Awards:

Dr. R. N. Philip

Invited to present a paper at the Sixth Annual Red Cross Scientific Symposium on "Transmissible Disease and Blood Transfusion", Washington, D.C., May 8-9, 1974.

Serial No. NIAID-183
1. Rocky Mountain Laboratory
2. Slow Viral Diseases Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Viruses as a probable cause of certain chronic diseases of man

Previous Serial Number: Same

Principal Investigator: W. J. Hadlow

Other Investigators: M. E. Bloom, B. W. Chesebro, J. E. Coe, T. A. Jackson, D. L. Lodmell, J. J. Munoz, J. L. Portis, and R. E. Race

Cooperating Units: Dr. C. M. Eklund, Hamilton, Montana (consultant)
USDA Animal and Plant Inspection Service, Veterinary Services: Dr. A. L. Klingsporn, Hyattsville, Md., and Mission, Texas

Man Years	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	14			14
Professional:	3 5/12			3 5/12
Other:	10 7/12			10 7/12

Project Description:

Objectives:

From a study of naturally occurring slow viral diseases of domestic animals, 1) to obtain an understanding of the mechanisms by which viruses cause protracted disease, and 2) to develop concepts and investigational procedures that will aid in determining whether viruses are causally related to some chronic diseases of man.

Methods employed:

Four diseases that are the models for continued study include: 1) scrapie, a subacute poliocencephalopathy of sheep, 2) a comparable neurologic disease of ranch mink, 3) Aleutian disease, a protracted systemic disease of ranch mink, and 4) progressive pneumonia, a chronic lymphoreticular proliferative pulmonary disease of sheep. Clinicopathologic, virologic, and immunologic features are studied to gain an understanding of the natural history and pathogenesis of each disease. Although no less important, study of the causative viruses is of secondary concern, largely because of lack of personnel.

1. Scrapie

a. Natural infection in sheep and goats. Several new observations support our previously stated view that lambs under conditions at the Scrapie Field Trial, Mission, Texas, most likely become infected via the oral route early in postnatal life. For example, virus has now been detected in 3 of 6 clinically normal Suffolk sheep 11 to 14 months old. In all three, virus was present mainly in the spleen, intestinal tract, and associated lymph nodes. Failure so far to detect virus in lambs younger than yearlings suggests a long eclipse phase of the infection. More sheep 6 to 18 months old will have to be examined for virus before we can ascertain whether the infection rate observed in yearlings is an indication of the incidence of disease in sheep 2 1/2 to 3 1/2 years old. In some families of Suffolks, the incidence of disease may reach 50% by this age, but it is usually much less. Thus, whether the incidence of infection in non-neural tissues of yearlings is greater than the usual incidence of disease in the same population of sheep 2 to 3 years later is still not clear; the occurrence of subclinical infection, therefore, remains unknown.

Our virologic findings in goats inoculated subcutaneously with mouse-adapted scrapie virus indicated that replication in non-neural tissues is limited; infection occurs mainly in lymph nodes and concentrations of virus are low. These and other observations led us to suggest that goat-to-goat transmission is rare or does not occur and that scrapie virus most likely does not become established in goats isolated from infected sheep. Recent virologic findings in 2 naturally infected goats from Mission, Texas, suggest that this may not be so. For example, in both goats, virus in moderately high concentrations was widespread in non-neural tissues, including the intestinal tract. The pattern of infection resembles that in Suffolk sheep rather than that in our experimentally infected goats. Whether this is typical of goats infected under natural conditions while cohabiting with infected Suffolk sheep remains to be determined. Strain of infecting virus may be important in explaining the differences observed.

b. Response of fetal sheep to scrapie virus. Results of viral assays are not yet available from the study mentioned last year to determine whether fetal sheep support replication of scrapie virus. Preliminary

results of immunologic responses of these fetuses, however, are of particular interest. Precolostral serum from 11 of 15 newborn lambs inoculated with a suspension of scrapie sheep brain at 74 to 103 days gestational age had immunoglobulin detectable by routine immunoelectrophoresis with anti-whole sheep serum. Mobility of the immunoglobulin was that of IgG and its location is compatible with the γG_1 subclass seen in mature sheep. Immunoglobulin was absent from the precolostral serum of one control lamb that had been inoculated as a fetus with a similar suspension of normal sheep brain. (By virtue of the syndesmochorial placenta of sheep, the fetal lamb does not receive immunoglobulins from its dam during pregnancy.) If the absence of immunoglobulins in lambs that receive normal sheep brain is borne out by examination of a larger number of such controls, the immunoglobulin response might be explained in several ways. 1) Scrapie virus is mitogenic and causes nonspecific stimulation of lymphocytes. 2) Scrapie virus has an adjuvant effect and the Ig occurs in response to antigens other than virus in the inoculum. 3) Scrapie virus is antigenic and the fetal immune response is directed to it. This study is being continued by Dr. Jackson.

c. Pathogenicity of scrapie virus for mink. Present evidence strongly suggests that mink encephalopathy may actually represent infection with scrapie virus. As mentioned last year, neurologic disease resembling encephalopathy occurred in mink inoculated intracerebrally with a suspension of brain from one of 2 Suffolk sheep affected with natural scrapie. A similar suspension from an affected Cheviot did not. To determine whether this is a common or a rare event, brain suspensions from other scrapie-affected sheep (6 Suffolk, 1 Targhee, and 1 Montadale) and from 2 affected goats were likewise inoculated intracerebrally into mink. Because most mink have been inoculated too recently, results are not yet available. Second passage in mink of the one Suffolk strain of virus pathogenic for this aberrant host shortened the incubation period from about 18 months to 8 to 10 months. So far, we do not know whether the first and second mink passaged scrapie virus is still pathogenic for mice.

2. Mink encephalopathy.

a. Course of infection in mink. Nothing new can be reported about the course of infection in mink. Conclusions given in last year's report still stand; the central nervous system is the only site where high concentrations of virus occur.

b. Response of sheep and goats to virus. As stated in previous reports, 10 of 19 goats became affected with a disease resembling scrapie 30 to 36 months after intracerebral inoculation with mink encephalopathy virus. By contrast, only 4 of 19 similarly inoculated Cheviot sheep have become affected with scrapie-like encephalopathy 40 to 70 months after inoculation. Several others are now (72 months after inoculation) clinically suspicious. Brain suspension of the first affected sheep caused typical encephalopathy in mink 7 to 8 months after intracerebral

inoculation. Mice inoculated with this suspension or a similar one from an affected goat have remained normal. Whether additional passages of encephalopathy virus in sheep or goats will make it pathogenic for mice is not known.

3. Aleutian disease (AD).

a. Response of pastel mink. As an extension of the studies reported last year on the varied response of pastel mink to Pullman AD virus, mink that remained clinically normal for 29 months after subcutaneous inoculation with a moderate amount of virus were challenged intraperitoneally with 10 LD_{50} of Porter isolate. Five of 7 became affected with AD and died or were killed when obviously sick 2 to 3 months after inoculation. The other two have elevated serum gamma globulin levels but are still clinically normal one year later. The large initial dose of virus obviously did not afford protection when the mink were challenged more than 2 years later with a much smaller amount of more virulent virus. In fact, quite the opposite occurred. Explanations for the unusually short course of the disease in the 5 challenged mink must be sought.

b. Oral inoculation of mink. As a necessary preliminary to a projected study mentioned last year, on the pathogenesis of the disease after oral inoculation of AD virus, starved mink were given serial 10-fold dilutions of virus in a bolus of feed. Surprisingly, the amount of virus required to cause disease by this route was at least 10^4 times that required by intraperitoneal inoculation. Pastel mink that proved unusually susceptible to intraperitoneal inoculation of the more virulent Porter virus also failed to become affected when given 10^5 intraperitoneal LD_{50} of virus orally. These observations suggest that oral exposure of adult mink to AD virus is not an effective mode of spread of the infection under natural conditions. This seems so despite the high titers of virus found in the intestine after subcutaneous inoculations. Vertical transmission, as suggested by the familial occurrence of the disease and by limited isolations of virus from fetuses, therefore, may account for most cases of the natural disease.

c. Studies with a third isolate of AD virus. Most of our information on AD has been obtained with the Pullman isolate. Comparative studies in the past 2 years have disclosed several biologic differences between this isolate and the Porter (Utah) isolate. Porter isolate replicates more quickly in sapphire mink and is much more virulent for pastel mink than the Pullman isolate. A third isolate was made during the summer of 1973 from a small group of sapphires on a local mink ranch. This isolate will allow us to extend our comparative studies on biologic properties of AD virus that may have a bearing on understanding the natural occurrence of the disease.

d. Cell culture studies. Despite continued effort to establish AD virus in a cell culture system, little new information can be reported. Virus was detected in the tenth in vitro passage of cells derived from bone marrow of a sapphire mink infected with the Pullman isolate. By contrast, cells derived from mesenteric lymph node of mink infected with either Pullman or Porter isolate have been uniformly negative for virus at various passage levels through the eleventh. Because recent reports indicate that AD virus will grow in certain stable lines of mammalian cells, use of explants of infected tissue seems a much less productive approach. Our finding that AD virus will at least persist for many passages in such explanted-derived cell cultures, nevertheless, may help explain what appear to be divergent conclusions about properties of AD virus purportedly established in cell culture by two different investigators.

e. Various studies on immunologic responses of mink infected with AD virus. Results of several collaborative studies are reported under Project NIAID-175.

4. Progressive pneumonia.

a. Course of experimental infection in goats. During the past year, 11 goats inoculated intravenously with a suspension of infected sheep lung were examined periodically for viremia by inoculation of cell cultures. Virus was not detected at any interval in any goat. These negative results may have been influenced by as yet to be surmounted technical difficulties in the cell culture assays. All goats have remained clinically normal.

b. Response of fetal sheep and goats to the virus. In a study similar to that reported for scrapie, fetal sheep and goats were inoculated intracerebrally with 0.2 ml of a suspension of infected sheep lung that contained 10^5 syncytial forming units per ml. Immunoglobulin like that found in newborn lambs inoculated in utero with scrapie virus was present in precolostral serum of 6 of 6 newborn kids and in the only newborn lamb tested. The significance of this finding remains to be determined, but the possible explanations mentioned for the fetal response to scrapie virus might apply here as well. The lambs and kids, which were allowed to live, have remained clinically normal.

Significance to biomedical research and the program of the Institute:

Comments offered repeatedly in past reports still seem appropriate in describing the significance of this project to biomedical research. As a project worthy of support by the Institute, it not only provides a point of departure for a fresh attack on disease problems heretofore considered outside the realm of infections but also complements more esoteric studies in molecular biology.

Proposed course of project:

Animal work will continue to provide the necessary operating basis to meet the objectives of the project. Nevertheless, some reduction in the amount of such animal work is contemplated to adjust to the reduction in personnel. The amount of collaborative studies will vary with the number of interested investigators and the extent that their needs for animal work can be met. Lack of personnel for laboratory virologic studies will continue to leave this important aspect of the project inadequately explored.

Some contemplated studies are:

1. More normal yearling Suffolk sheep from the Scrapie Field Trial will be examined for virus to provide a clearer understanding of the course of the natural scrapie virus infection.

2. The response of sapphire mink infected with AD virus in utero or in the early neonatal period will be re-examined to obtain a clearer understanding of the familial pattern of transmission observed in the natural disease.

3. Comparative studies on scrapie and mink encephalopathy viruses will be continued.

4. The pathogenic properties of the new isolate of AD virus for sapphire and pastel mink will be determined so that this isolate can be compared with the other two that behave differently.

5. If Border Leicester sheep, which supposedly are highly susceptible to progressive pneumonia virus, can be obtained, a renewed effort will be made to study the pathogenesis of the disease.

6. Tissue culture studies with AD virus will be continued, especially now that Porter seems to have outlined some of the conditions required for successful in vitro growth of the virus.

Honors and Awards:

Dr. W. J. Hadlow

Continued as Adjunct Professor of Veterinary Pathology, Washington State University, Pullman, Washington. In this capacity, served as member of graduate committee for Dr. Linda C. Cork who completed her Ph.D. thesis March 1974.

Accepted an appointment as a discussant in the Review Program in Veterinary Pathology sponsored by the Charles Louis Davis Foundation. (Convenes December 1975 in Chicago)

Publications:

Hadlow, W. J.: Myopathies of animals. In Pearson, C. M. and Mostofi, F. K. (Eds.): The Striated Muscle, IAP Monograph No. 12. Baltimore, Williams and Wilkins, 1973, pp. 364-409.

Eklund, C. M. and Hadlow, W. J.: Implications of slow viral diseases of domestic animals for human disease. Medicine 52: 357-361, 1973.

Lodmell, D. L., Bergman, R. K., and Hadlow, W. J.: Antibody-forming cells and serum hemolysin responses of pastel and sapphire mink inoculated with Aleutian disease virus. Infect. Immun. 8: 769-774, 1973.

Lavelle, G. C.: Multiplicity of scrapie virus in infected mouse spleen cells in vivo. Infect. Immun. 7: 918-921, 1973.

Cork, L. C., Hadlow, W. J., Crawford, T. B., Gorham, J. R., and Piper, R. C.: Infectious leukoencephalomyelitis of young goats. J. Infect. Dis. 129: 134-141, 1974.

In press:

Hadlow, W. J.: Myopathies of animals - A brief view. In Homburger, F. (Ed.): Advances in Cardiology, Vol. 13.

Eklund, C. M. and Hadlow, W. J.: Slow viral and related diseases. In Hubbert, W. T., McCulloch, W. F., and Schnurrenberger, P. R. (Eds.): Diseases Transmitted from Animals to Man (ed. 6).

Hadlow, W. J., Eklund, C. M., Kennedy, R. C., Jackson, T. A., Whitford, H. W., and Boyle, C. C.: Course of experimental scrapie virus infection in the goat. J. Infect. Dis.

Serial No. NIAID-184
1. Rocky Mountain Laboratory
2. Zoonoses Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Biological properties of Bordetella pertussis antigens

Previous Serial Number: Same

Principal Investigators: J. J. Munoz and R. K. Bergman

Other Investigators: None

Cooperating Units: Dr. Franklin Sogandares-Bernal, University of Montana, Missoula

Man Years	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	4 4/12			4 4/12
Professional:	1 6/12			1 6/12
Other:	2 10/12			2 10/12

Project Description:

Objectives:

1) To fully characterize biologically active substances from Bordetella pertussis with special reference to the histamine sensitizing factor (HSF), the protective antigen (PA) and the antibody stimulating substances (adjuvant) of these cells. 2) To study the mechanism of action of these substances.

Methods employed:

Standard immunological, pharmacological, biochemical, and bacteriological techniques, and physicochemical methods of fractionating biological materials are used.

Major findings:

1. Purification of HSF (Munoz). The method of purification of HSF described last year yields HSF in reasonable purity; however, it still contains traces of contaminants that have been difficult to remove. We have continued our efforts to obtain HSF free of contaminants.

2. Relationship of HSF to various biological activities of B. pertussis (Munoz and Bergman). It has been suspected for some time that many biological activities of pertussis vaccine are due to HSF, but absolute proof has not been obtained. To resolve this question, we tested various fractions obtained during the purification of HSF for the following biological activities known to be present in pertussis vaccine: 1) induction of lymphocytosis, 2) stimulation of γE globulin, 3) induction of hypoglycemia and hypoproteinemia, 4) acceleration of experimental allergic encephalomyelitis, and 5) mouse protective activity.

All fractions with HSF activity also possessed all other activities. The effective doses for the purest HSF preparation were: 1.1 μg induced histamine sensitivity; 0.44 μg induced lymphocytosis; 5 μg accelerated EAE; 1 μg stimulated γE production; 0.03 μg induced hypoglycemia; 0.8 μg induced hypoproteinemia; and 14 μg induced protection in mice. The finding that mouse protective activity of one fraction with relatively little HSF was as high as that of purified HSF indicates that more than one antigen may be involved in active immunization of mice against IC challenge with living B. pertussis. We must conclude that HSF is responsible for most biological activities present in whole cell vaccines.

3. Mechanism of action of HSF (Bergman and Munoz). We have suspected for some time that HSF may block β-adrenergic receptors and thus prevent epinephrine from acting to compensate for the vascular changes induced by vasoactive amines. Since histamine increases capillary permeability, this change is thought to affect HSF activity. It has been difficult, however, to demonstrate conclusively that tissue permeability occurs after administration of HSF. Employing the technique of Leibowitz and Kennedy (Imm. 22, 859, 1972) as reported last year, it has been possible to show systemic (muscle) and local (skin) increased vascular permeability to ^{131}I-HSA. The effect of other treatments and substances on vascular permeability also was studied. Adrenalectomy, x-irradiation, E. coli endotoxin, antilymphocyte serum and β-adrenergic blockade with propanolol were not effective in increasing vascular permeability. X-irradiation, however, greatly enhanced the subsequent effect of HSF.

The effect of catecholamines, histamine, serotonin and a combination of the latter two amines on localized permeability in the skin of normal

and HSF-treated mice was studied. HSF-treated animals had increased permeability at a skin site receiving histamine, serotonin or a combination of the amines. This permeability induced by histamine could be blocked in normal and in HSF-treated mice by catecholamines (isoproterenol, epinephrine, norepinephrine). Epinephrine and norepinephrine were ineffective, however, in preventing the permeability induced by serotonin, although isoproterenol was effective. The effectiveness of isoproterenol in preventing permeability changes either in the skin or in the thigh muscle is interesting because this catecholamine does not protect HSF-treated mice against death from histamine given systemically. The significance of these observations is not yet clear.

4. Histamine sensitization induced by Trichinella spiralis (Munoz, Bergman, and Sogandares-Bernal). T. spiralis infections in mice have been known to induce γE-globulin production with specificity to the T. spiralis antigens. B. pertussis extracts also increase γE to antigens given with it. For this reason it was thought that T. spiralis may also induce histamine sensitization in mice. The following observations have been made: 1) Oral infection of CFW male mice (only ones used thus far) with 50 to 100 larvae obtained from muscle of infected rats or mice induced a high degree of susceptibility to 0.5 mg of histamine given IP. 2) About the 12th day the larvae were deposited in the intestinal mucosa by the female, and sensitivity became apparent 13 to 15 days after infection. 3) The larvae were detected in the muscle of mice about the same time sensitivity to histamine was first detected. 4) The sensitivity to histamine persists for a long time. 5) The LD_{50} of histamine in infected mice is 0.18 mg per mouse, whereas that for normal mice is about 15 mg.

It is also interesting that the 72-hour PCA reactions (due to γE) can be induced with smaller amounts of antibody in normal mice than in infected mice and that sera of infected mice block PCA reactions when added to the test antibody before the antibody mixture is given to the recipient normal mouse. These observations indicate that the γE induced by the Trichinella infection may block the sensitization by γE having specificity to another antigen.

In preliminary work, no leukocytosis or increase in vascular permeability has been detected in infected mice. Furthermore, larval extracts given IP have not been found to be active in sensitizing mice to histamine.

Significance to biomedical research and the program of the Institute:

Because of its many activities, the HSF is a substance of great importance to biologists. One of its unique activities is increasing γE antibody production, with specificity to antigens given with it. This effect is similar to that induced by various trematode infections, such as T. spiralis. The fact that the serum of mice infected with T. spiralis

can "compete" with 72-hour PCA (γE antibodies) indicates that stimulation of nonspecific γE may be of some practical value in treating allergic diseases. This intriguing possibility for the application of HSF in allergies will be further investigated. The mode of action by which HSF induces production of γE and by which it enhances development of EAE are not known, but leads have been obtained that may prove highly significant. Increase in vascular permeability, in conjunction with the adjuvant effect of HSF, may be very important in the acceleration of EAE.

The protective antigen involved in immunization of children has not been yet identified. The mouse protective activity of B. pertussis cells may be due to many antigens, of which one may be HSF. Solution of this problem may provide practical answers for human vaccination.

Proposed course of project:

We will continue our efforts to fully characterize HSF. The histamine sensitization effect of T. spiralis and the similarities and differences to HSF will be studied. The mechanism of stimulation of γE and the effect of nonspecific γE on hypersensitivity reactions will be studied. The protective antigen(s) of B. pertussis will be investigated further.

Honors and Awards:

Dr. J. J. Munoz

Courtesy appointment as Faculty Affiliate, University of Montana.

On Editorial Board of Infection and Immunity.

Review papers for Journal of Infectious Diseases.

Invited to participate in the XXI Annual Symposium on Bacterial Toxins to be held in Hokano, Japan, August 1974.

Asked to participate in a workshop on mucosal diseases at the 2nd International Congress of Immunology to be held in Brignton, England, July 1974.

Asked with Dr. Bergman to write a monograph on the "Immunological Effects of B. pertussis" to be sponsored by the Center for Immunology, University of New York at Buffalo.

Serial No. NIAID-185
1. Rocky Mountain Laboratory
2. Epidemiology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Agents of the psittacosis-lymphogranuloma-trachoma group (Chlamydia)

Previous Serial Number: Same

Principal Investigators: R. N. Philip and R. K. Gerloff

Other Investigators: E. A. Casper and L. A. Thomas

Cooperating Units: Dr. Francis E. Gordon, Naval Medical Research Institute, Bethesda, Maryland (deceased)
Dr. David B. Lackman, Montana State Department of Health and Environmental Sciences, Helena
Dr. J. D. Oriel, University College Hospital, London, England
Dr. Peter Reeve, University of London, England

Man Years	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	2			2
Professional:	9/12			9/12
Other:	1 3/12			1 3/12

Project Description:

Objectives:

The general purpose of this project is to investigate relationships among the chlamydiae, to clarify the epidemiology of chlamydial diseases and determine their total importance to human health, and to apply technological advances to the laboratory study of these organisms.

Methods employed:

Serologic procedures, including the radioisotope precipitation (RIP) and micro-immunofluorescence (micro-IF) tests, have been evaluated recently as ancillary measures to isolation of chlamydiae in elucidating the extent and characteristics of human infection.

Major findings:

1. Serologic surveys. The parameters of RIP and micro-IF tests have been largely defined in serologic surveys of various population and age groups with varying exposure to chlamydial infection. To complete this phase of our investigation, the patterns of seroreactivity in older age groups were needed to complement our information which suggested a relationship in younger age groups of reactivity to the level of sexual activity. In 1973, sera obtained from 647 senior citizens who participated in a multiphasic screening program in Ravalli County were tested for micro-IF antibodies against various Chlamydia trachomatis immunotypes. The average age was 55 years and 37% were males. Overall, 12% had chlamydial antibodies and titers were generally low. There was no unusual age distribution, but the frequency of reaction among women was twice that among men. Average antibody titers were also distinctly higher among women. We have no explanation for these highly significant ($P < 0.001$) differences. Only 2% of persons had IgM antibodies and nearly all reactors were women. Nearly one half of IgM antibodies were specifically directed toward the TRIC, type A immunotype of C. trachomatis, which has never been associated with genital infection.

2. C. trachomatis antigens in RIP tests. In tests of C. trachomatis antisera, the RIP test with P^{32}-labeled meningopneumonitis particles measures only the chlamydial group antibody response. In an attempt to make the RIP test more specific for C. trachomatis responses, we prepared antigens from LGV (strain 404) and inclusion conjunctivitis (strain LB-1) chlamydiae grown in Hela 229 cells. The results were unsatisfactory (see 1972-1973 report). Because the LB-1 strain grows well in L cells, preparation of antigen from organisms grown in this cell line was attempted, in case the Hela cells previously used were contributing something deleterious to specificity. Results again were unsatisfactory. On the other hand, a fairly good RIP antigen was obtained from the C. psittaci D-383 strain of ornithosis.

3. Micro-IF antigens. Use of inclusion-containing whole cells from infected monolayers for antigen in the micro-IF test was investigated. Variation in intensity of staining from one infected cell to another, and even within a single inclusion, made it difficult to detect strain differences based on intensity of fluorescence. However, the lesser amount of cellular debris in tissue culture antigens, as compared with crude yolk sac antigens, contributed to unusually clear, bright pictures of fluorescing extracellular particles, some of which were always present in addition to

inclusions. Purified particle suspensions prepared from infected tissue culture cells disrupted at appropriate intervals should improve the precision of reading.

4. Miscellaneous. Others have reported steric hindrance of IgM immunofluorescence by avid IgG chlamydial antibody. A simple procedure for separating IgG and IgM immunoglobulins in test sera might increase the effectiveness of micro-IF assay for IgM antibody. Removal of as much as 90% of IgG antibody by several procedures (DEAE, sepharose immunoabsorbents) has not thus far unveiled IgM reactivity undetectable in whole serum.

Primary duck embryo cells were tested for susceptibility to 3 TRIC strains (UW-3, UW-6, and DC-049). All strains produced some inclusions, particularly UW-6, but growth was generally inferior to that in Hela 229 cells.

In completing investigations on toxicity of chlamydiae for cell monolayers preparatory to publication, a concerted effort was made to relate infectivity of a chlamydial suspension with its toxicity for L cells. With meningopneumonitis chlamydia as the prototype, it was found repeatedly that about 200 inclusion-forming units per cell were necessary to produce toxic effects, as measured by complete destruction of monolayers in 16 hours.

Significance to biomedical research and the program of the Institute:

Our knowledge of the importance of chlamydiae in human health, particularly agents transmitted directly from man to man, has lagged behind that concerning many other infectious agents. The particular problems have related to difficulties in establishing diagnoses and relating infection to disease. Laboratory procedures for isolating and identifying these agents have improved tremendously in the last decade, yet they still are not ideal. We have recently concentrated on developing, evaluating, and applying serologic techniques to study chlamydial infection, not as self-contained diagnostic procedures but as ancillary measures to be used in conjunction with isolation methods. We have shown that both the RIP test, because of its great sensitivity, and the micro-IF test, because of its immunotype responsiveness and capability to detect IgM antibodies indicative of primary infection, are useful epidemiologic procedures. From serosurveys we have attempted to gain some insight about the frequency of chlamydial infections in various population groups.

We have encouraged collaboration, particularly with British investigators, because they have been especially active in defining the relationship between chlamydial infection and such genito-urinary disease as nonspecific urethritis, a major problem both in Britain and in this country. Our serologic support has complemented their clinical acumen and their experienced use of isolation procedures to define genital TRIC infections.

Proposed course of project:

We plan to continue these studies, but with reduced emphasis. Our work in this area, at least in the near future, will be primarily collaborative, giving serologic support to Drs. Reeve and Oriel in their investigations of chlamydial and nonspecific urethritis. Concurrently we are "tying the loose ends" of preceding work in preparation for publication of findings to date (viz., application of RIP to epidemiologic study of chlamydial infection, comparison of RIP and micro-IF procedures, nature of chlamydial toxicity in cell cultures, immunoglobulin class of chlamydial antibody responses, and preparation of better micro-IF antigens).

The multi-team approach to determine etiologic relationships of chlamydiae to urethritis in several urban centers (see 1972-1973 report) has not materialized, and we feel that because of our remoteness from a large urban center, we are not in a favorable position to undertake a comprehensive epidemiologic study of this problem strictly on our own.

However, we have not become disinterested in this area of research and are prepared to resume full-scale activities if promising opportunity arises.

Publications:

Gordon, F. B., Quan, A. L., Steinman, T. I., and Philip, R. N.: Chlamydial isolates from Reiter's syndrome. Br. J. Vener. Dis. 49: 376-380, 1973.

Hill, D. A., Philip, R. N., Greaves, A. B., and Purcell, R. H.: Mycoplasmas recovered from patients with urethritis. Br. J. Vener. Dis. 49: 524-530, 1973.

In press:

Philip, R. N., Casper, E. A., Gordon. F. B., and Quan, A. L.: Fluorescent antibody responses to chlamydial infection in patients with lymphogranuloma venereum and urethritis. J. Immunol.

Serial No. NIAID-186
1. Rocky Mountain Laboratory
2. Medical Entomology and Acarology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The biology of microbial agents in arthropods

Previous Serial Number: Same

Principal Investigators: W. Burgdorfer and L. P. Brinton

Other Investigators: R. L. Anacker, B. N. Chaniotis, and L. A. Thomas

Cooperating Units: Dr. J. C. Cooney, Tennessee Valley Authority, Muscle Shoals, Alabama
Dr. V. S. Padbidri, WHO Fellow, Virus Research Center, Poona, India
Dr. D. J. Sexton, EIS Medical Epidemiologist, State Board of Health, Jackson, Miss.
Dr. R. B. Tesh, Pacific Research Station, Honolulu

Man Years	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	3 4/12			3 4/12
Professional:	1 4/12			1 4/12
Other:	2			2

Project Description:

Objectives:

To study dynamics of viral, rickettsial, and bacterial infections in arthropod vectors and the interactions between microorganisms and arthropod host, as reflected by changes in morphology, histology, and fine structure of arthropod tissues.

Research during the past year included 1) vesicular stomatitis virus (VSV) growth and cellular interaction in phlebotomine sandflies, 2) Colorado tick fever (CTF) morhpogenesis and localization in Dermacentor andersoni tissues, 3) study of rickettsia-like nonpathogenic microorganisms from field-collected Amblyomma americanum, D. andersoni, and D. variabilis, 4) characterization of a new spotted fever group rickettsia from the brown dog tick, Rhipicephalus sanguineus, from Mississippi, 5) comparative study of rickettsial agents and tick symbiotes in R. sanguineus, A. americanum, and Haemaphysalis leporispalustris, and 6) subcellular changes related to the spermatogenesis in D. andersoni.

Methods employed:

In addition to routine techniques of medical entomology, bacteriology, and virology, fluorescent antibody procedures are applied to studies of viral, bacterial, and rickettsial infections in arthropods. Electron microscopy (EM) is used to characterize subcellular structures of normal and infected arthropod tissues and to determine morphology of microorganisms during intracellular development.

Major findings:

1. The EM study of VSV in sandflies has progressed slowly because the majority of the 400 specimens submitted were found inadequately infiltrated with gluteraldehyde. In female flies, 24 hours after feeding on viremic hosts, VSV was consistently detected in gut epithelial cells, primarily in the peripheral cytoplasm adjacent to the basement membrane. In the cytoplasm of these cells, VSV was commonly surrounded by a membrane of apparent host cell origin. No VSV was found in blood meal residue in the gut lumen or in the peritrophic membrane. In fragments of sections obtained from 6-day post-fed females, VSV was observed in muscle tissues associated with the hypodermis. No virus was detected thus far in larval specimens derived from infected females.

2. Considerable efforts to demonstrate CTF virus in salivary glands, central ganglion, hypodermis, and ovarian tissues of experimentally infected D. andersoni failed.

3. Additional isolations of spotted fever-group-related rickettsiae were made from A. americanum collected in Tennessee and Alabama. Like the strains previously recovered, they were nonpathogenic for guinea pigs. Mild and transient infections in tunica vaginalis of meadow voles, however, could be established by injection of large concentrations of tissue culture-grown rickettsiae. Serial passage of infected tissues in meadow voles could not be accomplished.

In A. americanum the new isolates behaved like those previously studied; moderate rickettsial infections were present in all tissues, and

the organisms were passed via eggs to 100% of their progeny. No marked differences between these organisms and other spotted fever group agents could be seen by EM.

The bacillus-like, nonpathogenic microorganisms isolated from D. andersoni (western Montana, British Columbia, and Canada) and D. variabilis (Tennessee, Alabama, and South Carolina) failed to grow in mouse L cell monolayers. Their growth, however, has been achieved in chick embryo fibroblasts. Development is extremely slow and to date has been insufficient for preparing antigens for serologic comparison with pathogenic rickettsiae.

4. The spotted fever-group-related rickettsial agent detected in R. sanguineus from Mississippi (see NIAID-171) behaves in this tick like Rickettsia rickettsii does in D. andersoni, D. variabilis, or H. leporis-palustris. Infected female ticks collected from dogs were found to transmit this agent ovarially to 100% of their progeny. Organisms stained by conventional microscopy appear to be similar to symbiotes of R. sanguineus, but when examined by EM, their morphology was typical of rickettsiae and different from that of Wolbachia-like symbiotes. Tentative classification as a member of the spotted fever group was indicated by intracellular as well as intranuclear growth, cross reactions with FITC-labelled anti-R. rickettsii conjugates, and by immunity of guinea pigs that had been inoculated with microtus tissue suspensions and subsequently challenged with virulent R. rickettsii. On the other hand, fluorescent antibody staining patterns and physiologic behavior different from those of R. rickettsii suggest that this agent represents a new member of the spotted fever group.

5. Differentiation of rickettsial agents from Wolbachia-like symbiotes by conventional staining methods is difficult, particularly in ticks like R. sanguineus and A. americanum whose symbiotes appear indistinguishable from rickettsiae. During the past year, EM studies were conducted to establish the fine structure of symbiotes in these ticks as well as in H. leporispalustris. Observations made so far reveal morphologic characteristics unquestionably different from those of rickettsial agents.

6. Investigations into cellular changes occurring during spermatogenesis and the process of elongation during spermiogenesis in D. andersoni showed that upon completion of the second meiotic division early spermatids exhibit considerable cytoplasmic reorganization. This results in relocation of the nucleus and deep membranous cisternae to opposite ends of the cells. Cell elongation began with the nucleus confined to a polar position, cytoplasm was restricted around the periphery of the cell, and the central region of the cell contained further elongating membranous cisternae in a parallel array.

Significance to biomedical research and the program of the Institute:

Knowledge pertaining to the developmental fine structure of VSv-cell interaction in tissues of infected phlebotomine flies is necessary to fully understand the significance of these Diptera as vectors and reservoirs of this important agent. Observations made so far are too scanty to permit analysis as to localization and morphogenesis of VSV in this vector.

Failure to detect CTF virus by EM in experimentally infected D. andersoni is puzzling in view of findings by other investigators who described the morphogenesis of this agent in mouse brain and tissue cultures as similar to those of reoviruses. It appears that development of CTF virus in tick tissues differs from that in mice and tissue cultures or that virus concentrations are too low to be detectable by EM.

Significance of the nonpathogenic rickettsial agents isolated from A. americanum, D. andersoni, and D. variabilis cannot be assessed until their relationship to the spotted fever group rickettsiae, particularly to R. rickettsii, has been established. However, since all these ticks readily feed on man, it is essential that the rickettsia-like microorganisms are characterized and identified. Likewise, it is important to determine whether the agent in R. sanguineus causes disease in man.

Knowledge of morphology and development of tick symbiotes (Wolbachia) is required for studies of tick vector/pathogen relationships. Recently, a pathogen from H. leporispalustris was shown to cause cataract formation in suckling mice and was classified as a mycoplasma (Ziegel and Clark, 1974). This agent strongly resembles the Wolbachia-like symbiotes in H. leporis-palustris.

Proposed course of project:

Continuation of the EM study on morphogenesis and vector/relationship of VSV will depend on the availability of additional suitably prepared material.

Further attempts are in progress and will be continued to localize and demonstrate CTF virus in experimentally infected D. andersoni; emphasis will be placed on examination of muscle tissues which so far have not been examined.

Rickettsia-like microorganisms isolated from A. americanum, D. andersoni, and D. variabilis, as well as the spotted fever-group-related agent from R. sanguineus will be grown on various tissue culture systems to obtain sufficient material for purification by zonal rotor centrifugation. Purified preparations will then be subjected to biochemical and immunologic analyses to determine relationship of these agents to pathogenic rickettsiae.

A comparative study between the Wolbachia-like symbiotes from the rabbit tick, H. leporispalustris, and the suckling mouse cataract agent will be initiated.

Honors and Awards:

Dr. Willy Burgdorfer

Continued to serve as Faculty Affiliate for the Department of Zoology, University of Montana, Missoula.

Publications:

Burgdorfer, W., Brinton, L. P., and Hughes, L. E.: Isolation and characterization of symbiotes from the Rocky Mountain wood tick, Dermacentor andersoni. J. Invertebr. Pathol. 22: 424-434, 1973.

Burgdorfer, W., Schmidt, M. L., and Hoogstraal, H.: Detection of Trypanosoma theileri in Ethiopian cattle ticks. Acta Trop. 30: 340-346, 1973.

Oliver, J. H., Jr. and Brinton, L. P.: Sperm maturation in ticks: an example of capacitation in invertebrates? In Proceedings of 3rd International Congress of Acarology, Prague, 1971. Dr. W. Junk N.V., The Hague, The Netherlands, 1973, pp. 733-737.

In press:

Brinton, L. P., Burgdorfer, W., and Oliver, J. H.: Histology and fine structure of spermatozoa and egg passage in the female tract of Dermacentor andersoni Stiles (Acari-Ixodidae). Tissue and Cell.

Burgdorfer, W.: Spirochetal diseases. The relapsing fevers. In Hunter, G. W., Frye, W. W., and Swartzwelder, J. C. (Eds.): Manual of Tropical Medicine (ed. 5).

Serial No. NIAID-188
1. Rocky Mountain Laboratory
2. Medical Entomology and Acarology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Vector-pathogen relationships

Previous Serial Number: Same

Principal Investigator: C. E. Yunker

Other Investigators: C. M. Clifford, J. E. Keirans, R. A. Ormsbee, and L. A. Thomas

Cooperating Units: Dr. H. Hirumi, Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York
Dr. J. A. Howarth, University of California, Davis
Dr. M. N. Kaiser, NAMRU-3, Cairo, A.R.E.

Man Years	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	2			2
Professional:	5/12			5/12
Other:	1 7/12			1 7/12

Contract Support:

Contract Number	Project Officer (NIAID)	Contractor	Project Director	Annual Funding Level
PL-480 03-016-1	C. E. Yunker	NAMRU-3 Cairo, A.R.E.	M. N. Kaiser	$67,594

Project Description:

Objectives:

1) To investigate the host-range, cellular relationships, and growth dynamics in arthropods or their cells of pathogenic agents transmissible by vectors among man or animals, 2) to develop systems of vector or vector-related tissue and cell culture and to apply these systems to the study of arthropod-borne disease and vector-pathogen relationships, 3) to define the microenvironment to which pathogenic microorganisms are subjected when ingested by tick vectors, 4) to provide basic biochemical information necessary for the progress of developmental tick tissue culture studies.

Methods employed:

Disease agents of primary interest are arthropod-borne viruses and, to a lesser extent, rickettsiae, which are transmitted by Acarina and Insecta. Conventional basic techniques in laboratory arbovirology and experimental acarology and entomology are employed. A major effort involves development and use of arthropod tissue and cell cultures. Standard and specially devised biochemical and physiological techniques are used to determine the nature of tick body fluids. Resultant findings are employed in the preparation of tick tissue culture medium in efforts to improve the growth supporting characteristics of the medium.

Major findings:

1. Our previous findings demonstrated that the ability of an arbovirus to plaque in a mosquito cell-line correlates well with its serological group and vector-relationship. We then investigated some implication of these results for the recognition and classification of arboviruses.

Two strains of West Nile (WN) virus (typically mosquito-borne) isolated from ticks were compared with a mosquito isolate of WN in Aedes albopictus cells cultured in both fluid and agar overlay media. No differences in virus-growth dynamics or plaque morphology were seen, and this finding suggests that the actual source of the virus from the ticks was ingested host blood rather than infected tick tissues.

Japanese B encephalitis (JBE) virus and Tuleniy virus, both serologically closely related, were tested for their ability to grow in 2 lines of Culex tritaenorhynchus cells. This mosquito is the classical vector of JBE virus and cells derived from it should be able to support growth of Tuleniy virus if, as some workers have postulated, it is nothing more than a strain of JBE isolated from ticks. Both Hsu's and Schneider's lines of C. tritaenorhynchus supported rapid and abundant increase of the Nakayama and K29 strains of JBE virus, but failed to sustain Tuleniy virus (Three-Arch strain). This, together with our previous evidence that

Tuleniy virus will grow in tick cells but not in Ae. albopictus cells and results of transmission studies (see NIAID-181), indicates that it is not a mosquito-borne virus.

2. Twelve clones of Ae. albopictus cells were established. These will be tested for virus susceptibility and plaquing ability. It is known that virus production is limited to only a small proportion of the cells of the parent population yet, despite this, plaques are readily formed. Clonal populations might be expected to have differing susceptibilities to virus infection. Also, the superiority of Ae. albopictus cells for the primary isolation of certain viruses of low pathogenicity, e.g., dengue viruses, might further be improved.

3. Last year we reported that Rickettsia rickettsii and R. conori would cause plaque formation in Ae. albopictus cells. This year, in parallel plaque reduction neutralization tests, we found that significantly more protection was afforded Ae. albopictus cells than Vero cells by incubation of R. rickettsii with a homologous immune serum. Also, in contrast to antigens produced in Vero cells, which tend to contribute anti-complementarity activity, good complement-fixation (CF) antigens of R. rickettsii and R. conori were prepared from Ae. albopictus cell cultures. The yield of R. conori from one 30 ml culture flask was determined to be approximately equivalent to that from an infected egg.

4. In collaboration with Dr. J. A. Howarth, we attempted to isolate pathogenic agents from ticks collected in a focus of bovine abortion at Hopland, California. Seventy-two pools of ticks, including Dermacentor occidentalis, Ixodes pacificus, and Ornithodoros coriaceus were tested in Ae. albopictus and Vero cells, and suckling mice. Many of these were also tested in guinea pigs, Microtus, and embryonated eggs. Three pools of D. occidentalis yielded isolates of rickettsial organisms only in Vero and Ae. albopictus cells. When inoculated into guinea pigs, these isolates caused no symptoms or mild fever, but did induce CF antibodies to Rocky Mountain spotted fever (RMsf). Subsequently, these animals resisted challenge with virulent R. rickettsii; yolk sac antigens prepared from the isolates were positive in CF tests against RMsf antiserum. Indications are that the organism involved is a member of the RMsf group, but attempts failed to prepare a specific immune mouse sera against this and related agents endemic in the area. Selected bovine sera from aborted calves or mothers reacted in CF test only with Brucella, Francisella, or psittacosis antigens or were unreactive. The rickettsial isolates were passed in Vero cells and used to infect pregnant cows at the University of California (Davis); results are pending.

Three isolations of rickettsiae from bloods of local chipmunks were made in Ae. albopictus and Antheraea eucalypti cells. These agents produced plaques in both Ae. albopictus and Vero cells. Attempts to prepare a high

titered infectious stock in embryonated eggs were unsuccessful. Nevertheless, these results and the successful isolation of RMsf group organisms from California ticks demonstrate the utility of insect cells as systems for primary isolation of rickettsiae from field-collected materials.

5. Developmental tick tissue culture efforts have suffered greatly from our inability to hire an insectary attendant. Despite this, approximately 200 attempts were made to establish a continuous cell line from embryonated tick eggs, mostly of Dermacentor andersoni. Successes with other tick species reported by Russian and British workers could not be repeated with D. andersoni, but their techniques are promising and will be retested as ticks become available.

6. Progress in studies of tick biochemistry and physiology under PL-480 agreement No. 03-016-1 includes the publication of 10 research articles. Significant findings include:

a. Completion of a descriptive study of reproduction in parthenogenetic populations of Haemaphysalis longicornis. This disease vector and important pest of livestock was added to the study primarily because its unique mode of reproduction and the triploid nature of its genetic material make it of interest in developmental tick tissue culture efforts.

b. An experimental study of respiration in different physiological states of an ixodid tick has shown that oxygen consumption is highest in the mated, fed female, as opposed to the unmated, fed and unmated, partially fed female. The increase correlates in time with vitellogenesis. These facts are of significance in selecting physiological states of ixodid ticks for tissue culture purposes.

c. The relationship of feeding of ixodid and argasid ticks to oviposition, molting and protease activity were studied in detail. These factors conceivably influence the survival and multiplication of tick-borne pathogens in these vectors.

d. Studies of osmolarities for body fluids of argasid ticks and identities of fatty acids in fluids of ixodid ticks were completed. Results provide baseline data directly applicable to formulation of tissue culture media.

e. Qualitative and quantitative studies of nitrogenous excretion in argasid ticks continued. Knowledge of this process may reveal metabolic pathways and critical growth factors for tick cells, and possibly the influence of symbiotic microorganisms on tick metabolism.

f. As before, the need for new and more precise analytical techniques by which to study small quantities of tick fluids has resulted in publication of two valuable research notes on methodology.

7. We continued to supply, upon request, cell cultures, training, consultation, and information to institutions and to investigators, both here and outside the Institute.

In support of a study being made by Dr. R. Philip of Colorado tick fever (CTF) virus in human blood fractions (see NIAID-182) we made virus isolation attempts from serum, as well as from sonic-disrupted or whole washed erythrocytes. Isolation rates in Vero cells were comparable to those obtained in suckling mice or to infection rates as determined by immunofluorescent microscopy. Rates from sonicated erythrocytes were 9-20 times higher than from those with intact stroma.

In support of NIAID-181 we investigated the effect of heparin on isolation rate of certain tick-borne orbiviruses from blood samples. Earlier we had noted significant loss (± 3 logs) of CTF virus titers in heparinized plasma, as opposed to unheparinized serum, of viremic mouse bloods. This year tests were made with virus-infected guinea pig bloods and with standard, serum- or plasma-free virus diluents. Viruses used included CTF, Yaquina Head, Zirqa, and Sixgun City. In each case, incubation of the mixture with 1:20 (600 units/ml) heparin for 1 hour at 37° C followed by 24 hours storage at 4° C caused 2- to 10-fold loss of titer (PFU) compared with similarly treated samples lacking heparin. These findings indicate that certain tick-borne orbiviruses are inhibited by this sulfated polyanion, and that storage or transport of such viruses in blood samples should be done in the absence of heparin.

Significance to biomedical research and the program of the Institute:

These findings continue to demonstrate the utility of *in vitro* arthropod cell systems for the isolation and identification of arthropod-borne viruses and rickettsiae. In addition, they illustrate how such systems may be used to provide evidence with which to understand the cycle of such organisms in nature.

Biochemical and physiological studies of ticks provide a rational basis for development of tick tissue and cell cultures as well as insight into numerous factors affecting arthropod-vector potential and host-pathogen relationships.

Proposed course of project:

We will continue to investigate the growth dynamics of arboviruses in arthropod cells and to apply these systems as tools for the isolation and characterization of vector-borne pathogens. The PL-480 project will soon enter its final year, but a 3-year renewal will be proposed. Continued attempts to establish a line of tick cells depends upon the availability of live ticks. Continued lack of response to our repeated requests for an insectary attendant has seriously damaged this aspect of the project and affected other aspects of this and other projects as well.

Honors and Awards:

Dr. C. E. Yunker

By request, reviewed manuscripts for scientific publications: Journal of Parasitology, Journal of Medical Entomology, Federation of American Societies for Experimental Biology, Wilson Bulletin (Delaware Museum of Natural History), and Canadian Journal of Zoology, and in addition, for the Agricultural Research Service.

Editorial Board member, Journal of Medical Entomology.

Refereed research grant proposals for the National Science Foundation and the Environmental Protection Agency.

Nominated to the National Council on Systematics Collections and Environmental Quality, ASC.

Continued as Faculty Affiliate, Department of Zoology, University of Montana, Missoula.

Invited to participate in a symposium on host-parasite relationships sponsored by the N.Y. Academy of Sciences.

Publications:

Yunker, C. E. and Cory, J.: Growth of Rickettsia tsutsugamushi in insect cells. In Řeháček, J., Blaškovič, D., and Hink, W. F. (Eds.): Proceedings Third International Colloquium on Invertebrate Tissue Culture. Bratislava, Publishing House of the Slovak Academy of Sciences, 1973, pp. 451-457.

In press:

Cory, J., Yunker, C. E., Ormsbee, R. A., Peacock, M., Meibos, H., and Tallent, G.: Plaque assay of rickettsiae in a mammalian cell line. Appl. Microbiol.

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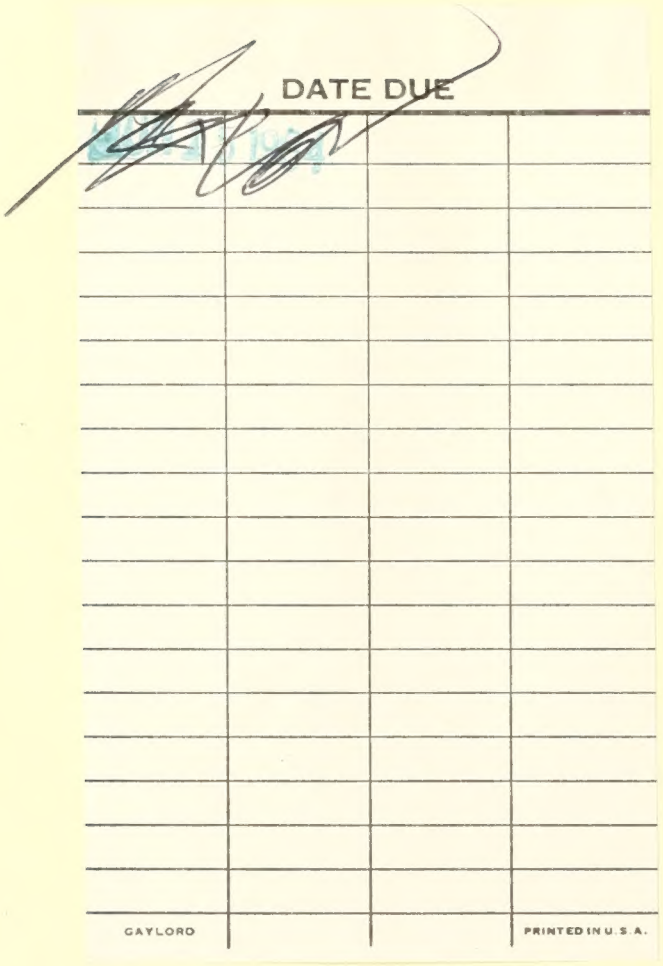

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